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<p>(21) International Application Number: PCT/DK97/00305 (22) International Filing Date: 7 July 1997 (07.07.97) (30) Priority Data: 0740/96 5 July 1996 (05.07.96) DK (71) Applicant (for all designated States except US): NOVO NORDISK A/S [DK/DK]; Novo Allé, DK-2880 Bagsværd (DK). (72) Inventor; and (75) Inventor/Applicant (for US only): CHRISTENSEN, Tove [DK/DK]; Novo Nordisk a/s, Novo Allé, DK-2880 Bagsværd (DK). (74) Common Representative: NOVO NORDISK A/S; Novo Allé, DK-2880 Bagsværd (DK).</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</p>
<p>(54) Title: A TRANSCRIPTION FACTOR</p> <p>(57) Abstract</p> <p>A transcription factor regulating α-amylase promoter initiated expression in filamentous fungi, especially in <i>Aspergillus</i>, DNA sequences coding for said factor, its transformation into and expression in fungal host organisms, and the use of said factor in such hosts for increasing the expression of a polypeptide of interest being produced by said host.</p>		

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Title: A Transcription FactorFIELD OF THE INVENTION

5 The present invention relates to a transcription factor found in filamentous fungi, especially in *Aspergillii*, DNA sequences coding for said factor, its transformation into and expression in fungal host organisms, and the use of said factor in such
10 hosts for increasing the expression of a polypeptide of interest being produced by said host.

BACKGROUND OF THE INVENTION

15 Transcription factors are well known proteins involved in the initiation of transcription. They have been studied intensively in many different organisms and have also been described in fungi. Dhawale and Lane (NAR (1993) 21 5537-5546) have recently compiled the transcription factors from fungi, including the
20 filamentous fungi.

Many of the transcription factors are regulatory proteins; they bind to the promoter DNA and either activate or repress transcription as a response to stimuli to the cell.

25

The expression of the α -amylase gene in *A. oryzae* is regulated in response to the available carbon source. The gene is expressed at its maximum when the organism is grown on starch or maltose (Lachmund et al. (1993) *Current Microbiology* 26 47-51;
30 Tada et al. (1991) *Mol. Gen. Genet.* 229 301-306). The expression of α -amylase is regulated at the transcriptional level as shown by Lachmund et al. (*supra*), which strongly suggests that transcription factors are involved in the regulation, but so far no gene for such a factor has been identified.

35

The promoter of the α -amylase gene has been studied by deletion analysis (Tada et al. (1991) *Agric. Biol. Chem.* 55 1939-1941;

Tsuchiya et al. (1992) *Biosci. Biotech. Biochem.* 56 1849-1853; Nagata et al. (1993) *Mol. Gen. Genet.* 237 251-260). The authors of these papers propose that a specific sequence of the promoter is responsible for the maltose induction. Nagata et al. (*supra*) used this sequence as a probe in a gel shift experiment to see whether any proteins from *A. nidulans* nuclear extracts were able to bind to the promoter sequence. Three such proteins were found, but no involvement of these proteins in expression was shown. None of the proteins have been purified or identified by other means. Their genes likewise remain unknown.

SUMMARY OF THE INVENTION

The present invention relates to a transcription factor regulating the expression of the α -amylase promoter in filamentous fungi.

Accordingly, in a first aspect the invention relates to a DNA construct comprising a DNA sequence encoding a transcription factor of the invention, which DNA sequence comprises

- a) the transcription factor encoding part of the DNA sequence cloned into plasmid pToC320 present in *E. coli* ToC1058, DSM 10666, or
- b) an analogue of the DNA sequence defined in a), which
 - i) is at least 60% homologous with the DNA sequence defined in a), or
 - ii) hybridizes with the same nucleotide probe as the DNA sequence defined in a), or
 - iii) encodes a transcription factor which is at least 50% homologous with the transcription factor encoded by a DNA sequence comprising the DNA sequence defined in a), or
 - iv) encodes a transcription factor which is immunologically reactive with an antibody raised against the purified transcription factor encoded by the DNA sequence defined in a), or
 - v) complements the mutation in ToC879, i.e. enables ToC879 to grow on cyclodextrin and produce lipase when transformed with said DNA sequence.

The full length genomic DNA sequence encoding a transcription factor has been derived from a strain of the filamentous fungus *Aspergillus oryzae* and has been cloned into plasmid pToC320
5 present in *E. coli* ToC1058, DSM 10666.

Said transcription factor encoding DNA sequence harboured in pToC320, DSM 10666, is believed to have the same sequence as that presented in SEQ ID NO: 1 and SEQ ID NO: 2. Accordingly,
10 whenever reference is made to the transcription factor encoding part of the DNA sequence cloned into plasmid pToC320 present in DSM 10666 such reference is also intended to include the transcription factor encoding part of the DNA sequence presented in SEQ ID NO: 1 and SEQ ID NO: 2.

15 Accordingly, the terms "the transcription factor encoding part of the DNA sequence cloned into plasmid pToC320 present in DSM 10666" and "the transcription factor encoding part of the DNA sequence presented in SEQ ID NO: 1 and SEQ ID NO: 2" may be used
20 interchangeably.

In further aspects the invention provides an expression vector harbouring the DNA construct of the invention, a cell comprising said DNA construct or said expression vector and a method of
25 producing a peptide exhibiting transcription factor activity, which method comprises culturing said cell under conditions permitting the production of the transcription factor.

Such a transcription factor of the invention will typically
30 originate from a filamentous fungus.

The term "filamentous fungus" is intended to include the groups Phycomycetes, Zygomycetes, Ascomycetes, Basidiomycetes and fungi imperfecti, including Hyphomycetes such as the genera
35 *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium* and *Humicola*.

The invention also relates to a method of producing a filamentous fungal host cell comprising the introduction of a DNA

fragment coding for any such factor into a filamentous fungus wherein an α -amylase promoter or a co-regulated promoter regulates the expression of a polypeptide of interest in a manner whereby said factor will be expressed in said fungus.

5

In a further aspect the invention relates to a method of producing a polypeptide of interest, the expression of which is regulated by an α -amylase promoter or a co-regulated promoter, comprising growing a filamentous fungal host cell as described
10 above under conditions conducive to the production of said factor and said polypeptide of interest, and recovering said polypeptide of interest.

Finally the invention relates to the use of said factor for
15 regulating the expression of a polypeptide of interest in a filamentous fungus.

In this context, regulation means to change the conditions under which the factor of the invention is active. This could mean
20 different pH, substrate, etc. regimes, whereby the resulting effect is an improved regulation of the expression of the protein of interest.

Furthermore, regulation also comprises events occurring in the
25 growth phase of the fungus during which the transcription factor is active. Depending on the circumstances, both advancing and/or postponing the phase wherein the factor is active may enhance the expression and thus the yield.

30 In addition, using standard procedures known in the art, the specific DNA sequences involved in the binding of a transcription factor may be identified, thereby making it possible to insert such sequences into other promoters not normally regulated by the factor and enabling those promoters to
35 be under the regulation of said factor.

BRIEF DESCRIPTION OF THE TABLES AND DRAWING

In the figures

Fig. 1 shows the structure of the plasmid pMT1657, the construction of which is described in Example 1;

Fig. 2 shows the structure of the plasmid pToC316, the construction of which is described in Example 1;

10 Fig. 3 shows the structure of the plasmid pToC320, the construction of which is described in Example 1;

Fig. 4 shows the structure of the plasmids pToC342 and pToC359, the construction of which are described in Example 3;

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Fig. 5 shows the structure of the plasmid pToC298, the construction of which is described in Example 4;

Fig. 6 shows the results of lipase production by a p960 transformant of *A. oryzae* IF04177 cultured in YP media containing 2% glucose (—■—) or 10% glucose (—◆—), in comparison to ToC1075 cultured in YP media containing 2% glucose (—□—) or 10% glucose (—◇—) and described in Example 4;

25 Fig. 7 shows the results of lipase production by ToC1139 cultured in YP media containing 2% glucose (—■—) or 10% glucose (—◆—), in comparison to ToC1075 cultured in YP media containing 2% glucose (—□—) or 10% glucose (—◇—) and described in Example 4; and

30

Fig. 8 shows the autoradiograph results of *A. niger* DNA digested with the following restriction enzymes: lane 2, *XbaI*; lane 3, *XmaI*; lane 4, *SalI*; lane 5, *HindIII*; lane 6, *EcoRI*; lane 7, *BglIII*; lane 8, *BamHI*; lanes 1 and 9 contain ³²P-labelled 1 DNA digested with *BstEII*. The experiment is described in Example 5.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect the invention relates to a DNA construct comprising a DNA sequence encoding a transcription factor regulating an α -amylase promoter, which DNA sequence comprises

- a) the transcription factor encoding part of the DNA sequence cloned into plasmid pToC320 present in *E. coli* ToC1058, DSM 10666, or
- b) an analogue of the DNA sequence defined in a), which
 - i) is at least 60% homologous with the DNA sequence defined in a), or
 - ii) hybridizes with the same nucleotide probe as the DNA sequence defined in a), or
 - iii) encodes a transcription factor which is at least 50% homologous with the transcription factor encoded by a DNA sequence comprising the DNA sequence defined in a), or
 - iv) encodes a transcription factor which is immunologically reactive with an antibody raised against the purified transcription factor encoded by the DNA sequence defined in a), or
 - v) complements the mutation in ToC879, i.e. enables ToC879 to grow on cyclodextrin and produce lipase when transformed with said DNA sequence.

As defined herein, a DNA sequence analogous to the transcription factor encoding part of the DNA sequence cloned into plasmid pToC320 present in *E. coli* ToC1058, DSM 10666, is intended to indicate any DNA sequence encoding a transcription factor regulating an α -amylase promoter, which transcription factor has one or more of the properties cited under (i)-(v) above.

The analogous DNA sequence may be isolated from a strain of the filamentous fungus *A. oryzae* producing the transcription factor, or another or related organism and thus, e.g. be an allelic or species variant of the transcription factor encoding part of the DNA sequence cloned into plasmid pToC320 present in DSM 10666.

Alternatively, the analogous sequence may be constructed on the basis of the DNA sequence presented as the transcription factor encoding part of SEQ ID NO: 1 and SEQ ID NO: 2, e.g. be a sub-
5 sequence thereof, and/or by introduction of nucleotide substitutions which do not give rise to another amino acid sequence of the transcription factor encoded by the DNA sequence, but which corresponds to the codon usage of the host organism intended for production of the transcription factor, or by
10 introduction of nucleotide substitutions which may give rise to a different amino acid sequence.

When carrying out nucleotide substitutions, amino acid residue changes are preferably of a minor nature, that is conservative
15 amino acid residue substitutions that do not significantly affect the folding or activity of the protein, small deletions, typically of one to about 30 amino acid residues; small amino- or carboxyl-terminal extensions.

20 Examples of conservative substitutions are within the group of basic amino acids (such as arginine, lysine, histidine), acidic amino acids (such as glutamic acid and aspartic acid), polar amino acids (such as glutamine and asparagine), hydrophobic amino acids (such as leucine, isoleucine, valine), aromatic
25 amino acids (such as phenylalanine, tryptophan, tyrosine) and small amino acids (such as glycine, alanine, serine, threonine, methionine). For a general description of nucleotide substitution, see e.g. Ford, et al., (1991), Protein Expression and Purification 2, 95-107.

30 It will be apparent to persons skilled in the art that such substitutions can be made outside the regions critical to the function of the molecule and still result in an active transcription factor. Amino acid residues essential to the
35 activity of the transcription factor encoded by a DNA construct of the invention, and therefore preferably not subject to substitution, may be identified according to procedures known in the art, such as site-directed mutagenesis or alanine-scanning

mutagenesis (cf. e.g. Cunningham and Wells, (1989), *Science* 244 1081-1085). In the latter technique mutations are introduced at every residue in the molecule, and the resultant mutant molecules are tested for biological activity (i.e. transcription factor regulating an α -amylase promoter) to identify amino acid residues that are critical to the activity of the molecule.

The homology referred to in (i) above is determined as the degree of identity between the two sequences indicating a derivation of the one sequence from the other. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., (1970), *Journal of Molecular Biology* 48 443-453). Using GAP with the following settings for DNA sequence comparison: GAP creation penalty of 5.0 and GAP extension penalty of 0.3, the coding region of the DNA sequence exhibits a degree of identity preferably of at least 60%, more preferably at least 70%, more preferably at least 80%, more preferably at least 90%, more preferably at least 95% with the transcription factor encoding part of the DNA sequence shown in SEQ ID NO: 1 and SEQ ID NO: 2.

The hybridization referred to in (ii) above is intended to indicate that the analogous DNA sequence hybridizes to the same probe as the DNA sequence encoding the transcription factor under certain specified conditions, which are described in detail in the Materials and Methods section hereinafter. The oligonucleotide probe to be used is the DNA sequence corresponding to the transcription factor encoding part of the DNA sequence shown in SEQ ID NO: 1 or SEQ ID NO: 2 or a fragment thereof.

The homology referred to in (iii) above is determined as the degree of identity between the two sequences indicating a derivation of the first sequence from the second. The homology may suitably be determined by means of computer programs known in the art such as GAP provided in the GCG program package (Needleman, S.B. and Wunsch, C.D., *supra*). Using GAP with the

following settings for transcription factor sequence comparison:
GAP creation penalty of 3.0 and GAP extension penalty of 0.1,
the transcription factor encoded by an analogous DNA sequence
exhibits a degree of identity preferably of at least 50%, more
5 preferably at least 60%, more preferably at least 70%, even more
preferably at least 80%, especially at least 90% with the
transcription factor encoded by a DNA construct comprising the
transcription factor encoding part of the DNA sequence shown in
SEQ ID NO: 2, e.g. with the amino acid sequence SEQ ID NO: 3.

10

In connection with property (iv) the immunological reactivity
may be determined by the method described in the Materials and
Methods section hereinafter.

15 In relation to the property (v) the complementation method is
described in Example 1 herein.

The DNA sequence encoding a transcription factor of the
invention can be isolated from the strain *Aspergillus oryzae* IFO
20 4177 using standard methods e.g. as described by Sambrook, et
al., (1989) Molecular Cloning: A Laboratory Manual. Cold Spring
Harbor Lab.; Cold Spring Harbor, NY.

General RNA and DNA isolation methods are also disclosed in WO
25 93/11249 and WO 94/14953, the contents of which are hereby
incorporated by reference. A more detailed description of the
complementation method is given in Example 1 herein.

Alternatively, the DNA encoding a transcription factor of the
30 invention may, in accordance with well-known procedures, be
conveniently isolated from a suitable source, such as any of the
below mentioned organisms, by use of synthetic oligonucleotide
probes prepared on the basis of a DNA sequence disclosed herein.
For instance, a suitable oligonucleotide probe may be prepared
35 on the basis of the transcription factor encoding part of the
nucleotide sequences presented as SEQ ID NO: 1 or any suitable
subsequence thereof, or on the basis of the amino acid sequence
SEQ ID NO: 3.

The invention relates specifically to a transcription factor regulating the expression of the α -amylase promoter in a filamentous fungus, which factor as indicated in Example 2 may even regulate the expression of other genes.

In this context the expression "filamentous fungus" is intended to include the groups Phycomycetes, Zygomycetes, Ascomycetes, Basidiomycetes and fungi imperfecti, including Hyphomycetes such as the genera *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium* and *Humicola*.

In this context the expression " α -amylase promoter" means a sequence of bases immediately upstream from an α -amylase gene which RNA polymerase recognises and binds to promoting transcription of the gene coding for the α -amylase.

As indicated, transcription factors are known from many organisms and it is therefore expected that similar or corresponding factors may be found originating from other fungi of the genera *Aspergillus*, *Trichoderma*, *Penicillium*, *Fusarium*, *Humicola*, etc., having an enhancing effect on the expression of a polypeptide being under the regulation of amylase promoters in any fungus belonging to any of these genera.

A comparison of the DNA sequence coding for the transcription factor regulating the α -amylase promoter has shown some degree of homology to a transcription factor (CASUCI) regulating the expression of glucosidase in *Candida* and to MAL63 of *Saccharomyces cerevisiae* as disclosed in Kelly and Kwon-Chung, (1992) *J. Bacteriol.* 174 222-232.

It is at present contemplated that a DNA sequence encoding a transcription factor homologous to the transcription factor of the invention, i.e. an analogous DNA sequence, may be obtained from other microorganisms. For instance, the DNA sequence may be derived by a similar screening of a cDNA library of another

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microorganism, such as a strain of *Aspergillus*, *Saccharomyces*, *Erwinia*, *Fusarium* or *Trichoderma*.

An isolate of a strain of *A. oryzae* from which the gene coding
5 for a transcription factor of the invention has been inactivated
has been deposited by the inventors according to the Budapest
Treaty on the International Recognition of the Deposit of
Microorganisms for the Purposes of Patent Procedure at the DSM,
Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH,
10 Mascheroder Weg 1b, D-38124 Braunschweig, DEUTSCHLAND.

Deposit date : 6 MAY 1996 (06.05.96)

Depositor's ref. : ToC879 = NN049238

DSM designation: *Aspergillus oryzae* DSM No.10671

15

The deposited strain *Aspergillus oryzae* DSM No.10671 can be used
to isolate a transcription factor according to the invention
from any strain of *Aspergillus oryzae* and any other fungal
strain having such a gene by complementation as described
20 hereinafter.

The expression plasmid pToC320 comprising the full length
genomic DNA sequence encoding the transcription factor of the
invention has been transformed into a strain of *E. coli*
25 resulting in the strain ToC1058, which has been deposited by the
inventors according to the Budapest Treaty on the International
Recognition of the Deposit of Microorganisms for the Purposes of
Patent Procedure at the DSMZ, Deutsche Sammlung von
Mikroorganismen und Zellkulturen GmbH., Mascheroder Weg 1b, D-
30 38124 Braunschweig, DEUTSCHLAND.

Deposit date : 6 MAY 1996 (06.05.96)

Depositor's ref. : ToC1058 = NN049237

DSM designation: *E. coli* DSM No.10666

35

According to the invention, factors of this type originating
from the species *A. oryzae*, *A. niger*, *A. awamori*, etc.,
especially *A. oryzae* IFO4177 are preferred.

The transcription factor of the invention has been found not only to be involved in the regulation of the α -amylase promoter, but also in the regulation of the glucoamylase promoter from *A. oryzae*.

Especially, the invention comprises any factor having an amino acid sequence comprising one or more fragments or combinations of fragments of the amino acid sequence depicted as SEQ ID NO: 3.

Truncated forms of the transcription factor may also be active. By truncated forms are meant modifications of the transcription factor wherein N-terminal, C-terminal or one or more internal fragments have been deleted.

A further aspect of the invention relates to a DNA sequence coding for any of these factors.

In this aspect the invention especially comprises any DNA sequence coding for one or more fragments of the amino acid sequence depicted as SEQ ID NO: 3.

More specifically the invention relates to a DNA sequence comprising one or more fragments or a combination of fragments of the DNA sequence depicted as SEQ ID NO: 1 and SEQ ID NO: 2.

According to a further aspect the invention relates to a method of producing a filamentous fungal host cell comprising the introduction of any of the above mentioned DNA fragments into a filamentous fungus wherein the α -amylase promoter or another co-regulated promoter regulates the expression of a polypeptide of interest in a manner whereby said factor will be expressed in said fungus.

The introduction of said DNA fragment may be performed by means of any well known standard method for the introduction of DNA

into a filamentous fungus, such as by use of an expression vector and host cells as described below.

Therefore, the invention also provides a recombinant expression
5 vector comprising the DNA construct of the invention.

The expression vector of the invention may be any expression vector that is conveniently subjected to recombinant DNA procedures, and the choice of vector will often depend on the
10 host cell into which it is to be introduced.

Thus, the vector may be an autonomously replicating vector, i.e. a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication,
15 e.g. a plasmid. Alternatively, the vector may be one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

20 In the expression vector, the DNA sequence encoding the transcription factor should either also contain the expression signal normally associated with the transcription factor or should be operably connected to a suitable promoter and terminator sequence. The promoter may be any DNA sequence which
25 shows transcriptional activity in the host cell of choice and may be derived from genes that are either homologous or heterologous to the host cell. The procedures used to ligate the DNA sequences coding for the transcription factor, the promoter and the terminator, respectively, and to insert them into
30 suitable vectors are well known to persons skilled in the art (cf., Sambrook, et al., supra).

Examples of suitable promoters for use in filamentous fungal host cells are, for instance, the *A. nidulans* ADH3 promoter
35 (McKnight, et al. (1985) *The EMBO J.* 4 2093-2099) or the *tpiA* promoter. Examples of other useful promoters are those derived from the gene encoding *Aspergillus oryzae* α -amylase, *Aspergillus niger* neutral α -amylase, *Aspergillus niger* acid stable α -

amylase, *Aspergillus niger*, *Aspergillus awamori*, or *Aspergillus*.
or *oryzae* glucoamylase (*gluA*), *A. oryzae* alkaline protease (*alp*),
A. oryzae nitrate reductase (*niaD*), *Aspergillus oryzae* triose
phosphate isomerase (*tpi*), *Aspergillus nidulans* acetamidase, or
5 an *Aspergillus* promoter coding for an amino acid biosynthetic
gene such as *argB*.

In yet another aspect the invention provides a host cell
comprising the DNA construct of the invention and/or the
10 recombinant expression vector of the invention.

Preferably, the host cell of the invention is a eukaryotic cell,
in particular a fungal cell such as a yeast or filamentous
fungal cell. In particular, the cell may belong to a species of
15 *Trichoderma*, preferably *Trichoderma harzianum* or *Trichoderma*
reesei, or a species of *Aspergillus*, most preferably *Aspergillus*
oryzae or *Aspergillus niger*. Fungal cells may be transformed by
a process involving protoplast formation and transformation of
the protoplasts followed by regeneration of the cell wall in a
20 manner known *per se*. The use of *Aspergillus* as a host microorga-
nism is described in EP 238 023 (Novo Nordisk A/S), the contents
of which are hereby incorporated by reference. The host cell may
also be a yeast cell, e.g. a strain of *Saccharomyces*, in
particular *Saccharomyces cerevisiae*, *Saccharomyces kluyveri* or
25 *Saccharomyces uvarum*, a strain of *Schizosaccharomyces* sp., such
as *Schizosaccharomyces pombe*, a strain of *Hansenula* sp., *Pichia*
sp., *Yarrowia* sp., such as *Yarrowia lipolytica*, or *Kluyveromyces*
sp., such as *Kluyveromyces lactis*.

30 The endogenous *amyR* gene of the host cell may be deleted or
inactivated by other means. The introduction of *amyR* control by
a heterologous promoter will then lead to a completely new
scheme of regulation of the α -amylase promoter. If, for
example, *amyR* is fused to the *A. oryzae niaD* promoter, the α -
35 amylase promoter will become inducible by nitrate. If, instead
of the *niaD* promoter, a *palC*-regulated promoter is used, the
activity of the α -amylase promoter will be regulated by pH.

The invention also comprises a method of producing a polypeptide of interest, whereby a host cell as described above is grown under conditions conducive to the production of said factor and
5 said polypeptide of interest, and said polypeptide of interest is recovered.

The medium used to culture the transformed host cells may be any conventional medium suitable for growing the host cells in
10 question. The expressed polypeptide of interest may conveniently be secreted into the culture medium and may be recovered therefrom by well-known procedures including separating the cells from the medium by centrifugation or filtration, precipitating proteinaceous components of the medium by means of a salt such
15 as ammonium sulphate, followed by chromatographic procedures such as ion exchange chromatography, affinity chromatography, or the like.

According to the invention the method may be used to produce a
20 polypeptide of interest that is a medicinal polypeptide, especially such medicinal polypeptides as growth hormone, insulin, blood clotting factor, and the like.

The method of the invention may also be used for the production
25 of industrial enzymes, such as proteases, lipases, amylases, glucoamylases, oxido reductases, carbohydrases, carbonyl hydrolases, cellulases, esterases, etc.

According to a further aspect of the invention said transcrip-
30 tion factor may be used for enhancing the expression of a polypeptide of interest in a filamentous fungus, such as a fungus of the genus *Aspergillus*, *Trichoderma*, *Penicillium*, *Fusarium*, *Humicola*, etc., especially of the species *A. oryzae*, *A. niger*, *A. awamori*, etc., and specifically *A. oryzae*.

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The transcription factor of the invention may thus be used to enhance the expression of a medicinal polypeptide, such as growth hormone, insulin, blood clotting factor, etc.

Also, the expression of industrial enzymes, such as proteases, lipases, amylases, glucoamylases, oxido reductases, carbohydrases, carbonyl hydrolases, cellulases, esterases, etc., may be enhanced by the use of the transcription factor of the invention.

The transcription factor may also be used to identify the sequences in the α -amylase promoter to which it binds. For example, this could be done by making a GST-fusion protein with the DNA binding domain of AmyR, such as the zinc finger, for production in *E. coli*. Such fusion proteins may be conveniently made using commercially available kits, for example, "The GST Gene Fusion Kit" from Pharmacia. The purified GST-fusion protein can then be used in conventional *in vitro* techniques such as gel shift assays or DNA footprint analyses (Kulmburg, P., et al. (1992) *Molecular and Cellular Biology* 12 1932-1939; Lutfiyya, L.L., and Johnston, M. (1996) *Molecular and Cellular Biology* 16 4790-4797). The identification of the AmyR binding site will make it possible to insert these sequences in other promoters not normally regulated by AmyR.

MATERIALS AND METHODS

25

Hybridization:

Suitable hybridization conditions for determining hybridization between a nucleotide probe and an "analogous" DNA sequence of the invention may be defined as described below. The oligonucleotide probe to be used is the DNA sequence corresponding to the transcription factor encoding part of the DNA sequence shown in SEQ ID NO: 1, i.e. nucleotides 1691..2676 + 2743..3193 + 3278..3653 in SEQ ID NO: 1, or a fragment thereof, e.g. nucleotides 1770-1800 in SEQ ID NO: 1.

35

Hybridization conditions

Suitable conditions for determining hybridization between a nucleotide probe and a homologous DNA or RNA sequence involves

pre-soaking of the filter containing the DNA fragments or RNA to hybridize in 5x SSC (standard saline citrate buffer) for 10 min, and prehybridization of the filter in a solution of 5x SSC (Sambrook, et al., *supra*), 5x Denhardt's solution (Sambrook, et al., *supra*), 0.5 % SDS and 100 µg/ml of denatured sonicated salmon sperm DNA (Sambrook, et al., *supra*), followed by hybridization in the same solution containing a random-primed (Feinberg, A. P. and Vogelstein, B. (1983) *Anal. Biochem.* 132 6-13), ³²P-dATP-labeled (specific activity > 1 x 10⁹ cpm/µg) probe for 12 hours at ca. 65°C. The filter is then washed two times for 30 minutes in 2x SSC, 0.5 % SDS at preferably not higher than 50°C, more preferably not higher than 55°C, more preferably not higher than 60°C, more preferably not higher than 65°C, even more preferably not higher than 70°C, especially not higher than 75°C.

Molecules to which the nucleotide probe hybridizes under these conditions are detected using a Phospho Image detector.

20 Immunological cross-reactivity:

Antibodies to be used in determining immunological cross-reactivity may be prepared by use of a purified transcription factor. More specifically, antiserum against the transcription factor of the invention may be raised by immunizing rabbits (or rodents) according to the procedure described by N. Axelsen et al. in: *A Manual of Quantitative Immuno-electrophoresis*, Blackwell Scientific Publications, 1973, Chapter 23, or A. Johnstone and R. Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, 1982 (more specifically pp. 27-31). Purified immunoglobulins may be obtained from the antisera, for example by salt precipitation ((NH₄)₂SO₄), followed by dialysis and ion exchange chromatography, e.g. on DEAE-Sephadex. Immunochemical characterization of proteins may be done either by Ouchterlony double-diffusion analysis (O. Ouchterlony in: *Handbook of Experimental Immunology* (D.M. Weir, ed.), Blackwell Scientific Publications, 1967, pp. 655-706), by crossed immuno-electrophoresis (N. Axelsen et al., *supra*, Chapters 3 and

4), or by rocket immunoelectrophoresis (N. Axelsen et al., op cit., Chapter 2).

EXAMPLES

5

EXAMPLE 1

Cloning of the *amyR* transcription factor from *A. oryzae*

amyR was cloned by complementation of an *A. oryzae* mutant strain unable to express two different proteins both under control of the TAKA-amylase promoter. The mutant *A. oryzae* strain ToC879 was made by mutagenesis of a strain, SRe440, containing a lipase (HLL) encoding cDNA under control of the TAKA promoter and one copy of the TAKA-amylase gene transcribed from its own promoter.

15 The mutant was identified and isolated by its amylase negative (amylase⁻) phenotype and subsequently shown to be lipase negative (lipase⁻) as well.

The strain ToC879 contains intact copies of both expression cassettes. The amylase⁻ phenotype makes ToC879 unable to grow on plates containing 1% cyclodextrin as the sole carbon source, while the parent strain SRe440 will grow on such plates.

ToC879 has been deposited at DSM under the name DSM No.10671.

25

amyR was isolated by co-transforming ToC879 with an *A. oryzae* cosmid library and an autonomously replicating pHelp1 based plasmid (D. Gems, I. L. Johnstone, and A. J. Clutterbuck (1991) Gene 98 61-67) carrying the *bar* gene from *Streptomyces* 30 *hygroscopicus* which confers resistance to glufosinate. The transformants were subjected to selection on plates containing cyclodextrin as the sole carbon source and screened for a concurrent reversion to the lipase⁻ phenotype.

35 The transforming DNA was rescued from colonies able to grow on cyclodextrin. Subcloning resulted in the isolation of a 4.3 kb DNA fragment able to complement both phenotypes of ToC879. The gene harboured on this fragment was named *amyR*.

Construction of the pHelp1 derivative pMT1657

A plasmid, pMT1612, was made by ligation (and subsequent transformation into *E. coli* DH5a) of the following four fragments:

- 5 i) the *E. coli* vector pToC65 (described in EP 531 372) cut with *SphI/XbaI*,
- ii) a PCR fragment (containing the *A. nidulans amdS* promoter) cut with *SphI/BamHI*,
- iii) a 0.5 kb *BamHI/XhoI* fragment from pBP1T (B. Staubinger et al., (1992) *Fungal Genetics Newsletter* 39 82-83) containing the
10 *bar* gene, and
- iv) a 0.7 kb *XhoI/XbaI* fragment from pIC AMG/Term (EP Application No. 87103806.3) containing the *A. niger* glucoamylase transcription terminator.

15

The PCR fragment containing the *amdS* promoter was made using the plasmid pMSX-6B1 (M. E. Katz et al., (1990) *Mol. Gen. Genet.* 220 373-376) as substrate DNA and the two oligonucleotides 4650 (SEQ ID NO: 4) and 4651 (SEQ ID NO: 5) as primers.

20

4650:	CTTGCATGCCGCCAGGACCGAGCAAG,	SEQ ID NO: 4
4651:	CTTGGATCCTCTGTGTTAGCTTATAG.	SEQ ID NO: 5

pMSX-6B1 contains an *amdS* promoter up mutation called I666.

25

pMT1612 was cut with *HindIII*, dephosphorylated and ligated to a 5.5 kb *HindIII* fragment from pHelp1 containing the AMAl sequence. The resulting plasmid, pMT1657 is self-replicating in *Aspergilli* and can be selected for by increased resistance to
30 glufosinate. pMT1657 is depicted in Fig. 1, wherein PamdS represents the *amdS* promoter of fragment ii) above, *bar* represents fragment iii) above, and Tamg represents fragment iv) above.

35 Construction of the cosmid library

A cosmid library of *Aspergillus oryzae* was constructed essentially according to the instructions from the supplier of the

"SuperCos1 cosmid vector kit" (Stratagene Cloning Systems, La Jolla CA, USA).

Genomic DNA of *A. oryzae* IFO4177 was prepared from protoplasts made by standard procedures (Christensen, T., et. al. (1988) *Biotechnology* 6 1419-1422).

After isolation the protoplasts were pelleted by centrifugation at 2500 rpm for 5 minutes in a Labofuge T (Heto); the pellet was then suspended in 10 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA, 100 µg/ml proteinase K and 0.5% SDS as stated in the manual from the Supercos 1 cosmid vector kit; the rest of the DNA preparation was done according to the instructions of the kit.

The size of the genomic DNA was analysed by electrophoresis using the CHEF-gel apparatus (Bio-Rad Laboratories, Hercules CA, USA). A 1% agarose gel was run for 20 hours at 200 volts with a 10-50 second pulse. The gel was stained with ethidium bromide and photographed. The DNA was 50->100 kb in size. The DNA was partially digested using *Sau3A*. The size of the digested DNA was 20-50 kb determined by the same type of CHEF-gel analysis as above. The CsCl gradient banded SuperCos1 vector was prepared according to the manual. Ligation and packaging were likewise performed as described in the manual.

After titration of the library, all of the packaging mix from one ligation and packaging was transfected into the host cells, XL1-Blue MR, and plated on 50 µg/ml ampicillin LB plates. Approximately 3800 colonies were obtained. Cosmid preparations from 10 colonies showed that they all had inserts of the expected size. The colonies were picked individually and inoculated in microtiter plate wells with 100 µl LB (100 µg/ml ampicillin) and incubated at 37°C overnight. 100 µl of 50% glycerol was added to each well, and the entire library was frozen at -80°C. A total of 3822 colonies were stored.

This represents the *A. oryzae* genome approximately 4.4 times. After picking the colonies the plates were scraped off, the scrape-off pooled and the total library was also stored in four pools as frozen glycerol stock. The four pools were named.
5 ToC901-ToC904.

The individually frozen colonies in the library were inoculated onto LB-plates (100 µg/ml ampicillin) by using a multipin device of 6 rows of 8 pins fitting into half a microtiter dish. Plates
10 were made containing colonies from all clones in the library.

The plates were incubated at 37°C overnight. Sterilized Whatman 540 filters cut to the size of a petri dish were placed upon the colonies which were incubated for two more hours at 37°C. The
15 filters were transferred to LB plates containing 200 µg/ml of chloramphenicol and the plates were incubated overnight at 37°C.

The next day the filters were washed twice in 0.5 M NaOH for 5 minutes, then twice in 0.5 M Tris-HCl (pH7.4) for 5 minutes and
20 then twice in 2x SSC for 5 minutes. The filters were wetted with ethanol and air dried.

Selection of *amyR* clones

Cosmid DNA was prepared from ToC901-904 and introduced into
25 ToC879 by co-transformation with pMT1657. The transformation procedure is described in EP Application No. 87103806.3. Approximately 8700 transformants were selected by resistance to 1 mg/ml glufosinate in minimal plates (Cove D.J. (1966) *BBA* 113 51-56) containing 1 M sucrose for osmotic stabilization and 10
30 mM (NH₄)₂SO₄.

Ten randomly chosen transformants were reisolated once on the same type of plates. Conidiospores from these 10 transformants were inoculated in minimal medium containing 1 mg/ml glufosinate
35 and grown at 30°C until enough mycelium for DNA preparation could be harvested. DNA was prepared as described in T. Christensen et al. (*supra*).

The uncut DNA was applied to a 0.7% agarose gel, and electrophoresis was performed, followed by Southern blotting. The blot was hybridized with a ³²P-labelled SuperCos1 specific DNA fragment. Each of the ten transformants showed a band with a higher mobility than the linear chromosomal DNA. Each of the bands also hybridized to a pHelp1 specific probe, indicating that the co-transformation frequency of the cosmid library was close to 100% and that the cosmids had integrated into the autonomously replication vector pHelp1.

10

The transformants were unstable as expected for pHelp1 transformants. Less than 10% of the conidiospores from a glufosinate resistant colony gave rise to glufosinate-resistant progeny.

15 Conidiospores from all the transformants were collected in 8 pools and plated on minimal plates (Cove D.J., *supra*) containing 1 mg/ml glufosinate, 10 mM (NH₄)₂SO₄ and 1% b-cyclodextrin (Kleptose from Roquette Frères, 62136 Lestem, France)

20 Four colonies were obtained from one of the pools and one from one of the other pools. Two of the colonies from the first pool were reisolated once on the same kind of plates.

Conidiospores from the reisolated colonies were plated on
25 minimal plates with either glucose or cyclodextrin as a carbon source and on glufosinate-containing plates. The glufosinate resistance and the ability to grow on cyclodextrin were both unstable phenotypes with the same degree of instability. This indicated that the gene conferring the ability to grow on
30 cyclodextrin was physically linked to pMT1657 in the transformants.

Colonies from the reisolation plates were cut out and were analysed by rocket immune electrophoresis (RIE) using an
35 antibody raised against the HLL lipase. The transformants gave a clear reaction with the antibody, while ToC879 colonies grown on maltose gave no reaction. This led to the conclusion that both the expression of amylase (i.e., growth on cyclodextrin) and

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lipase (i.e. antibody binding) had been restored in these transformants. The gene responsible for this phenotype was named *amyR*.

5 Isolation of the *amyR* gene

In order to rescue the *amyR* gene from the amylase⁺, lipase⁺ transformants of ToC879, two different approaches were used successfully.

- 10 DNA was prepared from mycelium grown in minimal medium with cyclodextrin as the carbon source.

In the first approach the DNA was packaged into λ -heads using the Gigapack[®] II kit from Stratagene in an attempt to rescue the
15 original cosmid out of the total DNA. The packaging reaction was incubated with XL1-Blue MR *E. coli* under the conditions specified by the kit supplier. The *E. coli* cells were plated on LB plates with 50 μ g/ml ampicillin. Two colonies appeared on the plates; the cosmids they contained were identical and named
20 ToC1012.

In the second approach the total DNA was used in an attempt to transform competent *E. coli* DH5a cells. Sixteen colonies were isolated and shown to contain six different plasmids by
25 restriction enzyme digest. Each of the plasmids was digested with *EcoRI* and subjected to Southern analysis. A ³²P-labelled probe of a mixture of pMT1657 and SuperCos1 was used to identify DNA fragments not part of any of these vectors. Two *EcoRI* fragments, approximately 0.7 and 1.2 kb in size, did not
30 hybridize to any of these probes. The 1.2 kb fragment was isolated, labelled with ³²P and used as a probe in a hybridization experiment with the filters containing the part of the cosmid library that gave rise to the original transformants. Six cosmids from the pool (ToC904), containing approximately
35 1000 clones did hybridize.

Of these, some were shown by restriction enzyme digestion to be identical, resulting in the isolation of four different cosmids.

All cosmids contained at least parts of the TAKA-amylase gene as well. The four cosmids and the cosmid ToC1012 were transformed into ToC879 by co-transformation with pMT1623, a pUC based plasmid that carries the *bar* gene under the control of the *A. oryzae tpi* promoter. Fifteen transformants from each co-transformation were isolated by resistance to glufosinate and tested for the ability to grow on cyclodextrin.

Eight transformants of ToC1012 and three transformants of one of the other cosmids, 41B12, were able to grow. None of the transformants of the other cosmids grew. That not all of the transformants of ToC1012 and 41B12 were able to grow is likely to be a reflection of the co-transformation frequency in each experiment. Colonies from the transformants growing on cyclodextrin were analysed by RIE, and showed that they all produced lipase.

DNA fragments obtained by digesting 41B12 with either *Bgl*III, *Hind*III or *Pst*I were cloned into pUC19 in order to subclone *amyR* from the cosmid. The subclones were transformed into ToC879 and the transformants analysed for the ability to grow on cyclodextrin and produce lipase as described above. As depicted in Fig. 2, one plasmid called pToC316 was shown to contain an approximate 9 kb *Hind*III fragment which was identified as containing *amyR*.

Further subcloning resulted in a plasmid called pToC320 containing a 4.3 kb *Hind*III/*Sac*I fragment, which is shown in Fig. 3 and was subsequently sequenced on an ABI DNA sequencer using both further subcloning and primer walking.

A DNA sequence of 3980 bp including the *amyR* gene is shown in SEQ ID NO: 1. The deduced amino acid sequence is shown in SEQ ID NO: 3 and reveals a Gal 4-type zinc finger sequence between amino acids 28-54. Such sequences are known to bind to DNA (Reece, R.J., and Ptashne, M. (1993) Science 261 909-910).

25

amyR maps close to one of the three amylase genes in IFO4177, since it was isolated from a cosmid also containing amylase-specific DNA fragments. Mapping of the cosmid showed that the α -amylase gene and amyR are 5-6 kb apart. Southern analysis of genomic DNA showed that only one copy of amyR is present in IFO4177, and confirmed that it maps close to one of the amylase genes.

Analysis of amyR cDNA

10 mRNA was made by the method of Wahleithner, J. A., et al. (1996, Curr. Genet. 29 395-403) from a culture of *A. oryzae* grown in maltose containing medium under conditions favorable for α -amylase production. Double stranded cDNA was made by standard procedures and used for PCR reactions with the following
15 primers:

oligodT primer:	TTTGTAAAGCT ₃₁	SEQ ID NO. 9
23087:	CCCCAAGCTTCGCCGTCTGCGCTGCTGCCG	SEQ ID NO. 6
20865:	CGGAATTCATCAACCTCATCAACGTCCTTC	SEQ ID NO. 7
20 20866:	CGGAATTCATCGGCGAGATAGTATCCTAT	SEQ ID NO. 8

A PCR reaction with the primers 20866 and 23087 resulted in a fragment of approximately 1.1 kb. The fragment was digested with *EcoRI* and *HindIII*; these restriction sites were incorporated
25 into the primers, and cloned into a pUC19 vector cut with the same enzymes.

The insert in the resulting plasmid was sequenced, the result located one intron in this part of the gene. The intron is
30 indicated in SEQ ID NO: 2.

Another PCR reaction with the oligodT primer and primer 20866 did not result in a distinct fragment. An aliquot of this reaction was used as the starting point for a new reaction with
35 the oligodT primer and the primer 20865, which resulted in a fragment of approximately 1.1 kb. This fragment was digested with *EcoRI* and *HindIII* and cloned into pUC19.

EXAMPLE 3

Overexpression of AmyR

A plasmid, pToC342, containing the coding region and 3' noncoding sequences of *amyR* fused to the promoter for the *A. oryzae tpi* gene was constructed. The *tpi* gene codes for triosephosphate isomerase, a constitutively expressed enzyme involved in primary metabolism. The *A. oryzae tpi* gene was isolated by crosshybridization with an *A. nidulans* cDNA clone according to the procedure of McKnight, G.L., et al, (1986, Cell 46 143-147). Sequencing led to identification of the structural gene. The promoter used was a fragment of approximately 700bp immediately upstream of the coding region. pToC342 was able to complement the mutation in ToC879. To pToC342 was further added the *A. oryzae pyrG* gene and the resulting plasmid, pToC359, was transformed into JaL250, a *pyrG* mutant of JaL228 described in patent application DK1024/96 filed 1996-09-19. Strains containing multiple copies of pToC359 were found to synthesise increased levels of glucoamylase.

20

Construction of pToC342 and pToC359

A PCR reaction was made with pToC320 as the template and the following primers:

25 8753 GTTTCGAGTATGTGGATTCC
8997 CGGAATTCCGATCCGAGCATGTCTCATTCTC

The resulting fragment was cut with *EcoRI*/*ApaI* to produce a fragment of approximately 180bp which was then cloned into pToC320 that had been digested with *EcoRI*/*ApaI*. The resulting plasmid, pToC336, was sequenced to confirm that the PCR fragment was intact. The 2.6kb *BamHI*/*SacI* fragment of pToC336 containing the coding region and the 3' untranslated sequence of *amyR* and an *EcoRI*/*BamHI* fragment of approximately 700bp containing the *tpi* promoter was cloned into *EcoRI*/*SacI* digested pUC19. The *BamHI* site downstream of the *tpi* promoter was introduced in vitro, whereas the *EcoRI* site is an endogenous site from the original *tpi* clone. The resulting plasmid, called pToC342, was

cut with *HindIII*, dephosphorylated and ligated to a 1.8 kb *HindIII* fragment containing the *A. oryzae pyrG* gene, resulting in a plasmid which was called pToC359. The structure of both pToC342 and pToC359 are shown in Fig. 4, wherein Ptpi represents the *tpi* promoter and TamyR represents the 3' noncoding region of *amyR*. The cloning of the *pyrG* gene has been previously described in WO 95/35385.

Expression in *A. oryzae* JaL250

JaL250 is a *pyrG* mutant of JaL228 selected by resistance to 5-fluoro-orotic acid. JaL228 has been described in patent application DK1024/96 filed 1996-09-19. JaL250 was transformed with pToC359 using standard procedures and by selecting for relief of uridine requirement. The transformants were reisolated twice through conidiospores and grown for four days in YP + 2% maltose at 30°C. Secreted glucoamylase was measured by the ability to cleave p-nitrophenyl α -D-gluco-pyranoside. The transformants had 5-31 arbitrary glucoamylase units/ml in the fermentation broth, while JaL228 had 2-3 units/ml. The best transformant was named ToC1200. Southern analysis showed that multiple copies of pToC359 had integrated into the genome of ToC1200. Because of the α -amylase promoter, ToC1200 may be used advantageously as a host strain for expression plasmids.

EXAMPLE 4

Carbon catabolite repression of the TAKA-promoter

The TAKA-amylase promoter is subject to carbon catabolite repression. In *Aspergilli* carbon catabolite repression is at least partially mediated via the transcriptional repressor CreA, a homologue to *S. cerevisiae* MIG1. The DNA binding sites in promoters under CreA control are known to be GC-rich and seemingly identical to the MIG1 sites in *S. cerevisiae*. The TAKA-amylase promoter contains several potential CreA binding sites. To determine whether this promoter is involved in carbon catabolite repression, three such sites were mutated, but provided only partial relief of carbon catabolite repression. In contrast, introduction of copies of constitutively expressed

AmyR in strains containing the modified promoter coupled to a reporter gene completely relieved repression of the reporter.

Construction of a CreA site deleted TAKA-amylase promoter

5 Three sites were identified as being potential CreA binding sites in the TAKA-amylase promoter by sequence comparison to known CreA and MIG1 sites. The resulting sites have the following sequences:

10 Site I CCCCGGTATTG
Site II CCCCGGAGTCA
Site III ATATGGCGGGT

The bases underlined were changed to A's because such changes
15 are known to destroy MIG1 binding sites. The substitutions were made using standard site-specific mutagenesis procedures. An expression vector, pToC297, containing the modified promoter and the 3' nontranscribed sequence of the glucoamylase gene from *A. niger* was constructed. pToC297 is identical to pToC68 described
20 in WO 91/17243 except for the changes in the promoter. Both plasmids have a unique *Bam*HI site between the promoter and the terminator.

Expression of a lipase regulated by a CreA⁻ TAKA-amylase 25 promoter

A *Bam*HI fragment of approximately 950bp containing the cDNA encoding a *Humicola lanuginosa* lipase was cloned into pToC297. (The cloning and expression of the *H. lanuginosa* lipase has been previously described in EP 305 216.) The resulting plasmid,
30 pToC298, was transformed into *A. oryzae* IFO4177 by co-transformation with the *A. nidulans amdS* gene, and its structure is shown in Fig. 5, wherein Ptaka-creA represents the CreA binding site deficient TAKA-amylase promoter. The transformants were reisolated twice through conidiospores and one such
35 transformant, ToC1075, which produces lipase, was chosen for further evaluation. ToC1075 and a p960 transformant of IFO4177 (previously described in EP 305 216) containing the lipase fused to the wild type TAKA-promoter were grown at 30°C in 10 ml YP

containing 2% or 10% glucose. Samples were taken daily for analysis of lipase in the fermentation broth. The lipase content was measured by rocket immune electrophoresis using a polyclonal antibody raised against purified lipase. Spent fermentation broth from *A. oryzae* IFO4177 did not react with the antibody. The glucose content of the fermentation broth was likewise measured daily using Tes-tape from Lilly.

On day one, glucose was detected in all cultures, but on day two glucose could be detected only in cultures originally containing 10%. The results of lipase production, shown in Fig. 6, indicate that the wild type promoter is repressed until glucose is no longer present. Thus, when the glucose becomes exhausted, lipase begins to accumulate. Fig. 6 also shows that the modified promoter is not as tightly regulated, as low levels of lipase are produced in the presence of glucose in the 10% glucose fermentation. Thus, there is partial glucose derepression seen in ToC1075.

20 Relief of carbon catabolite repression of lipase in ToC1075 by pToC342

ToC1075 was transformed with pToC342 by co-transformation with the *bar*-containing plasmid, pMT1623. Strains containing multiple copies of pToC342 and which retained the lipase expression cassette were identified by Southern blot analysis; one such strain was. ToC1075 and ToC1139 were grown at 30°C in 10 ml YP containing either 2% or 10% glucose, and samples were assayed daily for lipase and glucose. The lipase was measured by cleavage of para-nitrophenyl-butyrate. The glucose content was measured with Tes-tape from Lilly. The results, shown in Fig. 7, indicate that ToC1075, as before, provides partial relief of glucose repression while lipase production by ToC1139 is independent of the presence of glucose.

35 EXAMPLE 5

Southern analysis of *A. niger* for the *amyR* gene

The syntheses of α -amylase and glucoamylase in *A. niger*, as in *A. oryzae*, are regulated by the carbon source. It is therefore

likely that *A. niger* also contains an *amyR* gene. This hypothesis was tested by looking for cross-hybridization between the *A. oryzae amyR* gene and *A. niger* chromosomal DNA.

5 DNA was prepared from *A. niger* by conventional methods. The DNA was cut with *Bam*HI, *Bgl*III, *Eco*RI, *Hind*III, *Sal*I, *Xma*I or *Xba*I, and the resulting DNA fragments were separated by electrophoresis on an agarose gel. The DNA was then blotted onto a nitrocellulose membrane and hybridized with a ³²P-
10 labelled probe containing part of the structural *A. oryzae amyR* gene. The probe was made by PCR on pToC320 and starts at bp. no. 1683 and ends at bp. no. 2615 as shown in SEQ ID NO: 1. The hybridization was conducted in 10x Denhardt's solution, 5x SSC, 10mM EDTA, 1% SDS, 0.15 mg/ml polyA, 0.05 mg/ml yeast tRNA) at
15 50°C overnight. After hybridization the membrane was washed under conditions of increasing stringency and the radioactivity on the membrane analysed by a PhosphoImager. Figure 8 shows the result when the membrane had been washed in 2x SSC, 0.1%SDS at 58°C. Unique bands can be seen with several of the restriction
20 enzymes. Thus, the *A. niger amyR* gene can be cloned on the basis of this cross-hybridization result.

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WO 95/35385

WO 91/17243

30

EP 305 216

SEQUENCE LISTING

5 (1) GENERAL INFORMATION:

(i) APPLICANT:

10 (A) NAME: Novo Nordisk A/S
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(C) CITY: Bagsvaerd
(E) COUNTRY: Denmark
(F) POSTAL CODE (ZIP): DK-2880
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15 (H) TELEFAX: +45 4442 6080

(ii) TITLE OF INVENTION: A transcription factor

(iii) NUMBER OF SEQUENCES: 9

20 (iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
25 (D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

30 (A) LENGTH: 3980 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

40 (vi) ORIGINAL SOURCE:

(A) ORGANISM: *Aspergillus oryzae*

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

45 TCTAGACGGG CCACTGTGGG GTGCGGCAAG TTGATGCGG ACGGTGTGT AGTGTCTTCT 60
TTTAAAGAAC GGCACCGCTC TGGGCTCTCC GAACGGGAT TGTAGCTAGA TGATATGTC 120
50 TTGACGAACC AGGIGTCCAC GGCCTAATCC CTCACAATG ATGGCGCGTC CCGTTCCCAT 180
CGATTGTGTC TACCTGGCGT GCAAGGCATA ACATCGCGT CAAAGCTGCG AGGGGCATTC 240
CCTGCAATCT CTGACCATG AGAGGGGAAG CAAGTCACG TAGTTGCAAG GGTATAGGTC 300
55 CTAGCGAGCA ATGAGGTGGC TTCACCGTA CCGAGTGGG ACAGCATGAT CAGCGCTTTT 360
GGGAACTGA CGAAGAGTA CCGGTATAGC GCAAGATGG AGATGAATCT CTGCGGAGCA 420
60 AAGGAGGAGA CCGGAAAGA GTGTGTGAT TCTTGGGAG AGTACAGTA CTGCGGTGTC 480
CGGAAATGG AAAAGTCTT GAACATGCT GCGATCATC TGATATCGT ACGCTGATTG 540
GTGCATCGCC CGATAATGC CCGACAGAC GCTTGAAGCC TGAAAAGGTA GTATTCTGCG 600
65 AGAGATCCAT TCACAGAGT CAATCTGGC AAATACATG TTCCCGACCT CATATTCCAA 660
GGTGCGTAAA CCGCTCGGT GTGCGGCTGA GCGTTTCCA CGCATCTCT AGTGGTGGCA 720

	TGACGGGAGC ATCCGATGGC TTCCAGTATT GGGTGGTTGG GATGGACAAC AAGCTCCAAA	780
	TAAGGGGAAT TTGGCTTTGG TCACGGAATG AAGTCCCGT GGGGACGAGC GGCTCAGGCC	840
5	AGGCTAAGAG TCGAATATCG TCATAGACCT TGGCTCATG GGAGGTTGG AGGTGTTAAG	900
	ATCTCTTCA ATGCCATCA TTCTCTGTTT TGAOCTGGC TTCCGAGAG TGGTGGCTCC	960
10	CTTACATCC CACATGCTGG ATGCAAGCCT GTGGTAGCT GTTCTTTTCA GAAGTAGCAG	1020
	GCTAGGTCA CGATGAGCTG CCTTCAAAAC CTGGAATAAC CATTAGGTGA GACTGTCTA	1080
	CTTCTGAAT TGATCCCTGA CTAGAGTCTG CTCTAATAIG CTGTGTGGCA GGGCCGGTCC	1140
15	CCTGGGGT GCTAAGGCTG ATTATGACAC TCGTACAGT ATAACCCAGG GTGGCTATAG	1200
	ATTCCCTGCA TCTTCCAGGC TCCCTCAGAA CCTGATTCCA CCATTCTTAA GGGCCGGTGA	1260
	GCTCGATGG GGTATAATGG AGTTAACTAT AAACAGGACT CTACAAAGAA TCCCGATGIG	1320
20	AGTTTGAAC GAGTTGTAC CGATGGSTCC TCCATTGTGT TAGGAGTGAC GCTAGGCGAC	1380
	CTTTAGGCA CAGACTAAAC CAAGACAAAG ATGGAGTAGA CTCGAGTAG ATTAAITCCA	1440
25	ATCTCTTCC CAAAGTAAAG CGGGTTTTT TCAOCTGCA GCTCTTTTT TTCTTTTTT	1500
	CTTTTTTTT TTTTTTAT GTTCCCGAGA TTCTTTTCT TTTTCTTCAA TCTGAGGT	1560
	CTCAACGGT ATGGGACAC AGCCCGCTTC GCTATCCCTC GCTTTTAACT CGGCCATTCT	1620
30	TCTAGTTGCT CTGGGGGAT GGCATGATT CTAAGGCTC CACATCGGG AGATAGTATC	1680
	CTATCGAGC ATGTCTCATT CTCACACGA CATTCCCTCA ACATCCGAAA AGGAAATGGA	1740
35	GTCACCCCA GAAAGCCGC CTAACAGGC CTGGACAAT TGGGTGGAC GCAAAATCAA	1800
	GTGTCTAGA GAGCTTCAT GGGACAAGTG CCAGGCTCT CTCTCTCTCT GTTCTACAG	1860
	CGAGTCTCT CGTCCGAGG GCGCCAGTT CGGACGCTC TACCTCTCG CTCCATCCA	1920
40	TCCACTGGC TCACGACAC GTCTCTCAC CAAGGAATGG CTGCCCCAA ACCAGGGGC	1980
	TTGCCATTG GGTCCCGA CGTCTCGGC GTCCACGTA GGGGACGCC AGTATCTACA	2040
45	TCCAGACTTC TCGAGTGT TCACTGACT ACCACCCCA GATCTGTCT OCTCTCCGA	2100
	CTCGACAAAC TGGTATTGG ACTGTCCAC TATGGGGCA CTCCCGGCG CAGCGGTCT	2160
	GTCAGGCCA AACCTCTAG CCAATGCAA TGTCTCTC AAGTACCTGT TCCGATCAT	2220
50	GCCCGTGTG AGACAGGACC AGCTGCAGCA GCACTGCCAC CAGCGGAGC GCTTGTCTCC	2280
	CCACGCTAC GCTTTCATT CGCTCTATG CGCGGCCAG CACATCCAC TGAAGCTGGA	2340
55	CGTGCAGCA CGGGTCCCG AGGGGGCTTC CGCGGAGCC AGCCTGAGC GACATCTAT	2400
	GTGTCCGGA GAAGAATCC TGGCTGAAG CGTGGGGCA AGAAAGGAAT ACAAGTGGT	2460
	CGAGGAATT AACATGAAA ACTCTTAACT CTCTCTCTT CTCTTGGCG CCTACGGAA	2520
60	CCTAGACAGA CAGGATCAGS CCTGGTTCTA CTAATGTCAG ACCAGTCCA TGGTCTCAC	2580
	ACTAGGCTA CAACGGGAT CCACATCTC GAACTTAGC GTCGAGGAG CAGAAGAGAA	2640
65	AAGGAGATA TTCTGGCTCT TATTGTGAC AGAAAGGTAA GAAAGAAAA AACTTACTT	2700
	TCCCATCAC CACCACTAC CAAAATAAC ACGAAAAAC AGAGGCTAG CATTACAACA	2760
	AGCAAAACA GTCATGCTCC GCACTCCAT CCACAAACA CAGGTCTGT GCTCAGACA	2820

	CCCAATCCCTA GCTTACGGGT TCATCAACT CATCAAGTC TTGAAAAGC TCAGCCCAA	2880
5	TTCTTAAGAC TGGGTCTCG OGGGGGAG CAGGGAGAC GGGACGGG GGGTACTTC	2940
	TTCTATCAA TCCAGTCTG CCAAGCAAT CTCCCTGAG GGGTCTCG AGATCAGAA	3000
	AGTAGACATC CTCATCTC AGCAATGGT ACRAACCATG ATGTGGAAC TCTCATGAC	3060
10	CCAAGTCACA CAGGGGGCT CTGGGATGA GGGGTCTTC CCTTCCAC TGGGGTCT	3120
	AGTGGGCAAG GGGTCTCG GGGTCTCG GGGGATCC CAGGTGCTG TTGAGCTCA	3180
15	TGGTATCGA ATGGTAAGAA AGGAGCTTA CCTCATACA CCTGCTCA TCAGTCTC	3240
	CCCATCATCT ATACGGGCA TCTACAAA ACGGAGGAA CAAACTCT AGGAGCTGG	3300
	CACTGCTGA GGGAGCTCT GGGGCTGCT AAGCAAAA GGGGGGAC ACGTGGGGA	3360
20	ATGAGCATC GAGGGGGAG AACTCTCTG GGGATCTC ACAAGCTAT GGGAGTGG	3420
	GGTTCGCA TCAATCTCT TCCAGGGCT GGTGAGGAA AGTGGGGA TCAATGTT	3480
25	GGATGTTG CTTCATCA GTGATCTCT GCTTGGTT GGTGGGGG GGGTATTAT	3540
	GTTGGGAG GGTGAATCT GGTGTGTTT ATTGGGATC GGGATGATT TGCAAGAG	3600
	GGAGATGAG GGTGGGGAG GGTGTGTTT GGTGGGAG GAGATTGTT TTGAGGGGG	3660
30	CTCTTTCTT TTCTTTGT GGTGTGTTT GTTGGGTT TCTGGGGG GGGGGGTA	3720
	TATAGCTTG ACGATGGA TTGGGATGG GGTCTCTT GGTATATAT ATGGATGTT	3780
35	TTGTATATG TCCGCTGAG ACGGTCAAT GATGTGGGA TCAATCTT CTAGGACTC	3840
	GGAGCAGAG GTTGGGTT TGGGTATT CTGATGTA GATATATAG AATCAGTAA	3900
	TGATCATAT TGTACATAC TTAAGAAAG ATATGCTTG CACCGGATA TGCAATAGA	3960
40	AACTGGTCT TCATTCTAG	3980

(2) INFORMATION FOR SEQ ID NO: 2:

- | | |
|----|---|
| 45 | (i) SEQUENCE CHARACTERISTICS: |
| | (A) LENGTH: 3980 base pairs |
| | (B) TYPE: nucleic acid |
| | (C) STRANDEDNESS: single |
| | (D) TOPOLOGY: linear |
| 50 | (ii) MOLECULE TYPE: DNA (genomic) |
| | (iii) HYPOTHETICAL: NO |
| 55 | (iv) ANTI-SENSE: NO |
| | (vi) ORIGINAL SOURCE: |
| | (A) ORGANISM: <i>Aspergillus oryzae</i> |
| 60 | (ix) FEATURE: |
| | (A) NAME/KEY: exon |
| | (B) LOCATION: 1691..2676 |
| 65 | (ix) FEATURE: |
| | (A) NAME/KEY: intron |
| | (B) LOCATION: 2677..2742 |
| | (ix) FEATURE: |
| | (A) NAME/KEY: exon |
| | (B) LOCATION: 2743..3193 |

(ix) FEATURE:
 (A) NAME/KEY: intron
 (B) LOCATION:3194..3277

5 (ix) FEATURE:
 (A) NAME/KEY: exon
 (B) LOCATION:3278..3653

10 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION:join(1691..2676, 2743..3193, 3278..3653)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

15 TCTAGACCGG CCATGTGTGT GTCCGCCAAG TTGATTCCCG ACCGTGTGT AGTTGCTTCT 60
 TTAAAGAAC GGCACCCCTC TCCCGTCTCC GAACCGAAT TGTAGCTAGA TGTATATGTC 120
 20 TTGACGAACC AGGTGTCCAC GGCNAATCC CTCACAATTG ATGCCCGGTC CCGTTCCCAT 180
 CGATTGTGTC TACCTGGCGT GCAAGGCAAA ACATCCCGT CAAAGTCCG AGGGGCATTG 240
 CCTGCAATCT CTGACCATG AGAGGGGAG CAAGTCAAC TAGTTGCAAG GGTATAGGTC 300
 25 CTACGCAGCA ATGAGGTGGC TTCACCGGTA CGGATGGGG ACAGCATGAT CAAGCCTTTT 360
 GCGAAGTGA CGAAGAGTA CCGGTAAAC GAGGATGGG AGATGAATCT CTGCGAGCA 420
 30 AAGGACGAGA CCGGAAAGA GTGTGTGAT TCTTGGGAGC AGTTACAGTA CTTCGGTGTG 480
 CGGAATTTG AAAAGTCTCT GACCAATCT GCGATCATC TGATATCCCT ACCGTGATTG 540
 GTCCATCCCG CGATAAATGC CGACACGAC GCTTGAGGCC TGAAAAGGTA GTATTCTCG 600
 35 AGAGATCCAT TCACAGAGT CAATACGTC AATACATCG TTCCCACT CATATTCCAA 660
 GGTGCTAAA CCGCTCCGT GTGCGGTA GGTTTTCA GGCATCTCT AGTGTGCCA 720
 40 TGAGGGGAGC ATCGATGGC TTCCAGTATT GGTGTGTGG GATGGCAAC AAGCTCCAA 780
 TAAGGGGAAT TTGCTTTGG TCCAGGAATG AAGTCCCGT GGGACCCAG GCTCAGGCC 840
 AGGCTAAGAG TCGAATATCG TCATAGACT TCGCTCATG GAGGTTCGG AGGTGTACG 900
 45 ATCTCTTCA ATGCCATCA TTCTGTGT TTGCTGGC TTCCGAGAG TGTGCTCC 960
 CTTACATCC CACATGCTG ATGCAAGCT GTGGTACCT GTTCTTTCA GAAGTAGCAG 1020
 50 GCTAGTTCA GATGAGCTG CTTTCAAC CTGGAATAC CATTAGTGA GACTGTCTA 1080
 CTCTTGAAT TGAATCCGA CTAGATCTG CTCTAATG CTGTGCGCA CGCGGGTCC 1140
 CTTGGGGIT GCTAAGGCTG ATTTATGCAC TCGTACAGT ATAACCCAG GTGGCTATAG 1200
 55 ATTCCCTGCA TCTTCCAGC TCCCTACAA CTTGATCCA CATTCTTAA GCGCGGTA 1260
 GCTCGATGG GGTATATGG AGTTAACTAT AAACAGACT CTACACGAA TCCGATGTG 1320
 60 AGTTTGAAC GAGTTGTAC CGATGGTCC TCCATTGT TAGGATGAC GCTAGGGAC 1380
 CTTTAGGCA CAGACTAAC CAAGACAAAG ATGAGTAGA CTCAGGTAG ATTAATCCA 1440
 ATCTCTGCG CAAGTAAAG CGGGTTTTT TGAACCTGA GCTCTTTTT TTCTTTTTT 1500
 65 CTTTTTTT TTTTTTAT GTTCCCGA TTCTTTTT TTCTTTCA TCTGAGTT 1560
 CTCACCGTG ATGGGACAC AGCCCGTTC GCTATCCCT GCTTTTACG CGGCATCT 1620

38

	TCTAGTTGCT CTGGGGGAT GCCATGATTT CTAAAGGCTC CACATGGGG AGATAGTATC	1680
5	CTAATGGAGC ATG TCT CAT TCT CCA ACC GAC ATT CCC TCA ACA TCC GAA Met Ser His Ser Pro Thr Asp Ile Pro Ser Thr Ser Glu 1 5 10	1729
10	AAG GAA ATG GAG TCA ACC CCA GAA AAG CCG CCT AAA CAG GCC TGC GAC Lys Glu Met Glu Ser Thr Pro Glu Lys Pro Pro Lys Gln Ala Cys Asp 15 20 25	1777
15	AAT TGC CGT CGA CGC AAA ATC AAG TGT TCT AGA GAG CTT CCA TGC GAC Asn Cys Arg Arg Arg Lys Ile Lys Cys Ser Arg Glu Leu Pro Cys Asp 30 35 40 45	1825
	AAG TGC CAG CGT CTT CTT CTC TCC TGT TCC TAC AGC GAC GTG CTC CGT Lys Cys Gln Arg Leu Leu Leu Ser Cys Ser Tyr Ser Asp Val Leu Arg 50 55 60	1873
20	CGC AAG GGC CCC AAG TTC CGC AGC CTC TAC CCT CTC GCT CCC ATC CAT Arg Lys Gly Pro Lys Phe Arg Thr Leu Tyr Pro Leu Ala Pro Ile His 65 70 75	1921
25	CCA CTC GGC TCA CGA CCA CGT CCT CTC ACC AAG GAA TGG CTG CCC CCA Pro Leu Ala Ser Arg Pro Arg Pro Leu Thr Lys Glu Trp Leu Pro Pro 80 85 90	1969
30	AAC CCA GGG GCT TGC CAT TTG GCG TCC CCG AGC TCT CCG CGC TCC ACC Asn Pro Gly Ala Cys His Leu Ala Ser Pro Thr Ser Pro Pro Ser Thr 95 100 105	2017
35	GTA GCG GAC GGC CAG TAT CTA CAT CCA GAC TTC TCG GAG TCG TTC ACT Val Ala Asp Ala Gln Tyr Leu His Pro Asp Phe Ser Glu Ser Phe Thr 110 115 120 125	2065
	CGA CTA CCA CCC CCA GAT CTC GTC TCC TCT CCC GAC TCG ACA AAC TCG Arg Leu Pro Pro Pro Asp Leu Val Ser Ser Pro Asp Ser Thr Asn Ser 130 135 140	2113
40	CTA TTC GAC TCG TCC ACT ATC GGC GCA CTC CCC GCG CCA CGC CGT CTG Leu Phe Asp Ser Ser Thr Ile Gly Ala Leu Pro Ala Pro Arg Arg Leu 145 150 155	2161
45	TCG AGC CCA AAC CTT CTA GGC CAT GTC AAT GTC TTC CTC AAG TAC CTG Ser Thr Pro Asn Leu Leu Ala His Val Asn Val Phe Leu Lys Tyr Leu 160 165 170	2209
50	TTC CCG ATC ATG CCC GTC GTG AGA CAG GAC CAG CTG CAG CAG GAC TGC Phe Pro Ile Met Pro Val Val Arg Gln Asp Gln Leu Gln Gln Asp Cys 175 180 185	2257
55	CAC CAG CCG GAG CGC TTG TCT CCC CAA CGC TAC GCT TTC ATT GGC GCT His Gln Pro Glu Arg Leu Ser Pro Gln Arg Tyr Ala Phe Ile Ala Ala 190 195 200 205	2305
	CTA TCC GCG GGC ACG CAC ATC CAA CTG AAG CTG GAC GGT GCA CCA CCG Leu Cys Ala Ala Thr His Ile Gln Leu Lys Leu Asp Gly Ala Ala Pro 210 215 220	2353
60	GGT CCC GAG GCG GCT TCC GCG CCA GGC AGC CTC GAC GGA CAT CCT ATG Gly Pro Glu Ala Ser Ala Arg Ala Ser Leu Asp Gly His Pro Met 225 230 235	2401
65	TTG TCG GGA GAA GAA CTC CTG GCT GAA GGC GTG CGC CCA AGA AAG GAA Leu Ser Gly Glu Glu Leu Leu Ala Glu Ala Val Arg Ala Arg Lys Glu 240 245 250	2449
	TAC AAC GTG GTC GAC GAA ATT AAC ATG GAA AAC CTC CTA ACC TCC TTC	2497

39

	Tyr Asn Val Val Asp Glu Ile Asn Met Glu Asn Leu Leu Thr Ser Phe	
	255 260 265	
5	TTT CTC TTC GCC GCC TAC GGA AAC CTA GAC AGA CAG GAT CAG GGC TGG Phe Leu Phe Ala Ala Tyr Gly Asn Leu Asp Arg Gln Asp Gln Ala Trp 270 275 280 285	2545
10	TTC TAC CTA TGT CAG ACC ACG TOC ATG GTC TTC ACA CTA GGC CTA CAA Phe Tyr Leu Cys Gln Thr Thr Ser Met Val Phe Thr Leu Gly Leu Gln 290 295 300	2593
15	CGG GAA TCC ACA TAC TCG AAA CTA AGC GTC CAG GAA GCA GAA GAG AAA Arg Glu Ser Thr Tyr Ser Lys Leu Ser Val Glu Glu Ala Glu Glu Lys 305 310 315	2641
20	AGG AGA GTA TTC TGG CTC TTA TTC GTC ACA GAA AG GTAAGAAAAG Arg Arg Val Phe Trp Leu Leu Phe Val Thr Glu Arg 320 325	2686
25	AAAAAATCT ACTTTCCCAA TCACCAACAC GTACCAAAAA TAACAGAAA AACCG A GOC TAC GCA TTA CAA CAA GCA AAA CCA GTC ATG CTC GGC AAC TCC ATC Gly Tyr Ala Leu Gln Gln Ala Lys Pro Val Met Leu Arg Asn Ser Ile 330 335 340 345	2743
30	CAC AAA CCA CAG GTC CTG TGC TCA GAC GAC CCA ATC CTA GGC TAC GGC His Lys Pro Gln Val Leu Cys Ser Asp Asp Pro Ile Leu Ala Tyr Gly 350 355 360	2791
35	TTC ATC AAC CTC ATC AAC GTC TTC GAA AAG CTC AGC CCA AAT CTC TAC Phe Ile Asn Met Ile Asn Val Phe Glu Lys Leu Ser Pro Asn Leu Tyr 365 370 375	2839
40	GAC TGG GTC TOC GCC GGC GGC AGC AGC GCA GAC GGC GAC CCC CCG CCT Asp Trp Val Ser Ala Gly Gly Ser Ser Ala Asp Gly Asp Pro Pro Pro 380 385 390	2887
45	ACT TCT TCT ATC CAA TOC AGT CTC GGC AAG CAA ATC TOC CTC GAG GGC Thr Ser Ser Ile Gln Ser Ser Leu Ala Lys Gln Ile Ser Leu Glu Gly 395 400 405	2935
50	GTC TOC GAG ATC CAG AAA GTA GAC ATC CTC ATC ACT CAG CAA TGG CTA Val Ser Glu Ile Gln Lys Val Asp Ile Leu Ile Thr Gln Gln Trp Leu 410 415 420 425	2983
55	CAA ACC ATG ATG TGG AAA CTC TOC ATG ACC CAC GTC ACA CAG CCC GGC Gln Thr Met Met Trp Lys Leu Ser Met Thr His Val Thr Gln Pro Gly 430 435 440	3031
60	TCT GGC GAT GAC GGC GTT CTC OCC TTC CAC CTG CCC GTG CTA GTC GGC Ser Arg Asp Asp Ala Val Leu Pro Phe His Leu Pro Val Leu Val Gly 445 450 455	3079
65	AAG GGC GTC ATG GGC GTC ATC GGC GGC GCA TOC CAA GGT GCT GTT GAC Lys Ala Val Met Gly Val Ile Ala Ala Ala Ser Gln Gly Ala Val Asp 460 465 470	3127
	GCT CAT GGT ATC GGA ATG GTAAGAAAG GACCTTAOCT CATCACACC Ala His Gly Ile Gly Met 475	3175
	TCCTCATCA GTCACTOCCC ATCATCTATA CCCCATCT AACAAAAACC GCAG GAA Glu 480	3223
	CAA AAA CTC TAC GAC CTC GGC ACC TOC GTA GGC GAC GTC TOC GGC TOC Gln Lys Leu Tyr Asp Leu Gly Thr Ser Val Ala Asp Val Ser Arg Ser 485 490 495	3280
		3328

40

	CTA AGC ACA AAA GCC GGC CAC CAC CTC GGC GAA TGG ACC ATC GAC CCC Leu Ser Thr Lys Ala Ala His His Leu Ala Glu Ser Thr Ile Asp Pro 500 505 510	3376
5	CGA GAA CTC CTC TGG GGC ATT CTC ACA ACC CTA TOC CGA ATC GGC GGT Arg Glu Leu Leu Trp Gly Ile Leu Thr Thr Leu Ser Arg Ile Arg Gly 515 520 525	3424
10	TOC CAA TCA TAC CTC TTC CCA GCG CTC GTC GAG CAA AGT CGA GGC ATC Ser Gln Ser Tyr Leu Phe Pro Ala Leu Val Glu Gln Ser Arg Gly Ile 530 535 540	3472
15	ATC AGT TTC GAC TGT TGG CTT TOC ATC AGT GAC TTT CTG CCT TGG TTT Ile Ser Phe Asp Cys Ser Leu Ser Ile Ser Asp Phe Leu Pro Ser Phe 545 550 555 560	3520
20	GGT GGG CCG CCG GCT ATT ATG TGG CCG AGG GGT GAA TCT GGG TTT GAT Gly Gly Pro Pro Ala Ile Met Trp Arg Thr Gly Glu Ser Gly Phe Asp 565 570 575	3568
25	TTA TTG GGG ATC GCG GAT GAT TTG CAA GAG AGG GAG AAT GAG GGT GCG Leu Leu Gly Ile Ala Asp Asp Leu Gln Glu Arg Glu Asn Glu Gly Gly 580 585 590	3616
30	GAG GGG ATT GTG GTG GCT GCG GAG GAG ATT TGG TTT TGAGGGGGCT Glu Gly Ile Val Val Ala Gly Glu Ile Ser Phe 595 600	3662
35	CTTTCTTTT TCTTTTGGG TGTTTGTGT TGGTGGTTC TGGGGGGGG GGGGTGTATA TACGCTTGAC GATGTGCATT GGCATTGGGG TTCTACTGG TATAATATAT GATTTGTTT GTATATAGTC CGCTGGAC GGTGCAATGA TTGGGGGATC AATCACTTCT TAGGACTGG AGCACAGGT GTGGTTCCT GGGTATCTCT GAGTATGAGA TTATATAGAA TCAGTTATG ATCATTTATG TACATACCTT AAAGAAAGAT ATGCTTGGCA CCGGATATG ACAATAGAAA ACTGGTCTTC ATTCAGA	3722 3782 3842 3902 3962
40		3980

(2) INFORMATION FOR SEQ ID NO: 3:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 604 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

Met	Ser	His	Ser	Pro	Thr	Asp	Ile	Pro	Ser	Thr	Ser	Glu	Lys	Glu	Met
1				5				10				15			
Glu	Ser	Thr	Pro	Glu	Lys	Pro	Pro	Lys	Gln	Ala	Cys	Asp	Asn	Cys	Arg
20				25				30							
Arg	Arg	Lys	Ile	Lys	Cys	Ser	Arg	Glu	Leu	Pro	Cys	Asp	Lys	Cys	Gln
35				40				45							
Arg	Leu	Leu	Leu	Ser	Cys	Ser	Tyr	Ser	Asp	Val	Leu	Arg	Arg	Lys	Gly
50				55				60							
Pro	Lys	Phe	Arg	Thr	Leu	Tyr	Pro	Leu	Ala	Pro	Ile	His	Pro	Leu	Ala
65				70				75						80	
Ser	Arg	Pro	Arg	Pro	Leu	Thr	Lys	Glu	Trp	Leu	Pro	Pro	Asn	Pro	Gly
85				90				95							

41

Ala Cys His Leu Ala Ser Pro Thr Ser Pro Pro Ser Thr Val Ala Asp
100 105 110

5 Ala Gln Tyr Leu His Pro Asp Phe Ser Glu Ser Phe Thr Arg Leu Pro
115 120 125

Pro Pro Asp Leu Val Ser Ser Pro Asp Ser Thr Asn Ser Leu Phe Asp
130 135 140

10 Ser Ser Thr Ile Gly Ala Leu Pro Ala Pro Arg Arg Leu Ser Thr Pro
145 150 155 160

Asn Leu Leu Ala His Val Asn Val Phe Leu Lys Tyr Leu Phe Pro Ile
165 170 175

15 Met Pro Val Val Arg Gln Asp Gln Leu Gln Gln Asp Cys His Gln Pro
180 185 190

20 Glu Arg Leu Ser Pro Gln Arg Tyr Ala Phe Ile Ala Ala Leu Cys Ala
195 200 205

Ala Thr His Ile Gln Leu Lys Leu Asp Gly Ala Ala Pro Gly Pro Glu
210 215 220

25 Ala Ala Ser Ala Arg Ala Ser Leu Asp Gly His Pro Met Leu Ser Gly
225 230 235 240

Glu Glu Leu Leu Ala Glu Ala Val Arg Ala Arg Lys Glu Tyr Asn Val
245 250 255

30 Val Asp Glu Ile Asn Met Glu Asn Leu Leu Thr Ser Phe Phe Leu Phe
260 265 270

35 Ala Ala Tyr Gly Asn Leu Asp Arg Gln Asp Gln Ala Trp Phe Tyr Leu
275 280 285

Cys Gln Thr Thr Ser Met Val Phe Thr Leu Gly Leu Gln Arg Glu Ser
290 295 300

40 Thr Tyr Ser Lys Leu Ser Val Glu Glu Ala Glu Glu Lys Arg Arg Val
305 310 315 320

Phe Trp Leu Leu Phe Val Thr Glu Arg Gly Tyr Ala Leu Gln Gln Ala
325 330 335

45 Lys Pro Val Met Leu Arg Asn Ser Ile His Lys Pro Gln Val Leu Cys
340 345 350

50 Ser Asp Asp Pro Ile Leu Ala Tyr Gly Phe Ile Asn Leu Ile Asn Val
355 360 365

Phe Glu Lys Leu Ser Pro Asn Leu Tyr Asp Trp Val Ser Ala Gly Gly
370 375 380

55 Ser Ser Ala Asp Gly Asp Pro Pro Pro Thr Ser Ser Ile Gln Ser Ser
385 390 395 400

Leu Ala Lys Gln Ile Ser Leu Glu Gly Val Ser Glu Ile Gln Lys Val
405 410 415

60 Asp Ile Leu Ile Thr Gln Gln Trp Leu Gln Thr Met Met Trp Lys Leu
420 425 430

65 Ser Met Thr His Val Thr Gln Pro Gly Ser Arg Asp Asp Ala Val Leu
435 440 445

Pro Phe His Leu Pro Val Leu Val Gly Lys Ala Val Met Gly Val Ile
450 455 460

42

Ala Ala Ala Ser Gln Gly Ala Val Asp Ala His Gly Ile Gly Met Glu
 465 470 475 480
 5 Gln Lys Leu Tyr Asp Leu Gly Thr Ser Val Ala Asp Val Ser Arg Ser
 485 490 495
 Leu Ser Thr Lys Ala Ala His His Leu Ala Glu Ser Thr Ile Asp Pro
 500 505 510
 10 Arg Glu Leu Leu Trp Gly Ile Leu Thr Thr Leu Ser Arg Ile Arg Gly
 515 520 525
 Ser Gln Ser Tyr Leu Phe Pro Ala Leu Val Glu Gln Ser Arg Gly Ile
 530 535 540
 15 Ile Ser Phe Asp Cys Ser Leu Ser Ile Ser Asp Phe Leu Pro Ser Phe
 545 550 555 560
 20 Gly Gly Pro Pro Ala Ile Met Trp Arg Thr Gly Glu Ser Gly Phe Asp
 565 570 575
 Leu Leu Gly Ile Ala Asp Asp Leu Gln Glu Arg Glu Asn Glu Gly Gly
 580 585 590
 25 Glu Gly Ile Val Val Ala Gly Glu Glu Ile Ser Phe
 595 600

(2) INFORMATION FOR SEQ ID NO: 4:

30

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 26 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

35

(ii) MOLECULE TYPE: primer 4650

(iii) HYPOTHETICAL: YES

40

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

45 CTTGCATGCC GCCAGGACCG AGCAAG 26

(2) INFORMATION FOR SEQ ID NO: 5:

50

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 26 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

55

(ii) MOLECULE TYPE: primer 4651

(iii) HYPOTHETICAL: YES

60

(iii) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

65 CTTGGATCCT CTGTGTTAGC TTATAG 26

(2) INFORMATION FOR SEQ ID NO: 6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 30 base pairs

43

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

5 (ii) MOLECULE TYPE: primer
 (iii) HYPOTHETICAL: YES
 (iii) ANTI-SENSE: NO
10 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:
 CCCCAAGCTT CGCCGCTCTGC GCTGCTGCCG30
15 (2) INFORMATION FOR SEQ ID NO: 7:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 29x base pairs
20 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: primer
25 (iii) HYPOTHETICAL: YES
 (iii) ANTI-SENSE: NO
30 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:
 CGGAATTCAT CAACCTCATC AACGTCTTC 29
35 (2) INFORMATION FOR SEQ ID NO: 8:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 29 base pairs
40 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: primer
45 (iii) HYPOTHETICAL: YES
 (iii) ANTI-SENSE: NO
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:
50 CGGAATTCAT CGGCGAGATA GTATCCTAT 29
55 (2) INFORMATION FOR SEQ ID NO: 9:
 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 41 base pairs
 (B) TYPE: nucleic acid
60 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear
 (ii) MOLECULE TYPE: primer
 (iii) HYPOTHETICAL: YES
65 (iii) ANTI-SENSE: NO
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

WO 98/01470

PCT/DK97/00305

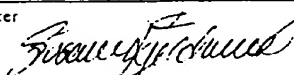
44

TTTGTAAAGC TTTTTTTT TTTTTTTT TTTTTTTT T

41

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

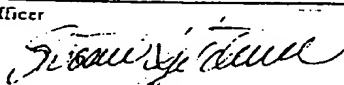
(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>11</u> , line <u>11-13</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH	
Address of depositary institution (including postal code and country) Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY	
Date of deposit 1996-05-10	Accession Number DSM 10671
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
<p>Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.</p>	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	
<p>For receiving Office use only</p> <p><input checked="" type="checkbox"/> This sheet was received with the international application</p> <p>Authorized officer </p>	<p>For International Bureau use only</p> <p><input type="checkbox"/> This sheet was received by the International Bureau on:</p> <p>Authorized officer</p>

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description on page <u>11</u> , line <u>31-34</u>	
B. IDENTIFICATION OF DEPOSIT Further deposits are identified on an additional sheet <input type="checkbox"/>	
Name of depositary institution DEUTSCHE SAMMLUNG VON MIKROORGANISMEN UND ZELLKULTUREN GmbH	
Address of depositary institution (including postal code and country) Mascheroder Weg 1b, D-38124 Braunschweig, GERMANY	
Date of deposit 1996-05-10	Accession Number DSM 10666
C. ADDITIONAL INDICATIONS (leave blank if not applicable) This information is continued on an additional sheet <input type="checkbox"/>	
Until the publication of the mention of grant of a European patent or, where applicable, for twenty years from the date of filing if the application has been refused, withdrawn or deemed withdrawn, a sample of the deposited microorganism is only to be provided to an independent expert nominated by the person requesting the sample (cf. Rule 28(4) EPC). And as far as Australia is concerned, the expert option is likewise requested, reference being had to Regulation 3.25 of Australia Statutory Rules 1991 No 71. Also, for Canada we request that only an independent expert nominated by the Commissioner is authorized to have access to a sample of the microorganism deposited.	
D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)	
E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)	
The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")	

For receiving Office use only <input checked="" type="checkbox"/> This sheet was received with the international application Authorized officer 	For International Bureau use only <input type="checkbox"/> This sheet was received by the International Bureau on: Authorized officer
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CLAIMS

1. A transcription factor regulating the expression of an α -amylase promoter in filamentous fungus.
- 5 2. The factor of claim 1 originating from a fungus of the genus *Aspergillus*, *Trichoderma*, *Penicillium*, *Fusarium*, *Humicola*, etc.
- 10 3. The factor of claim 2 originating from the species *A. oryzae*, *A. niger*, *A. awamori*, especially *A. oryzae* IFO4177.
4. The factor of claim 3 having an amino acid sequence comprising one or more fragments of the amino acid sequence depicted as SEQ. ID. No 3.
- 15 5. A DNA construct having a DNA sequence coding for the factor of any of the claims 1 to 4.
- 20 6. The DNA sequence of claim 5 having a DNA sequence comprising one fragment or a combination of fragments of the DNA sequence depicted as SEQ ID NO:1.
7. A DNA construct comprising a DNA sequence encoding a transcription factor exhibiting activity in regulating the expression of an α -amylase promoter in a filamentous fungus, which DNA sequence comprises
 - a) the transcription factor encoding part of the DNA sequence cloned into plasmid pToC320 present in *E. coli* ToC1058, DSM 30 10666, or
 - b) an analogue of the DNA sequence defined in a), which
 - i) is at least 60% homologous with the DNA sequence defined in a), or
 - ii) hybridizes with the same nucleotide probe as the DNA sequence defined in a), or
 - 35 iii) encodes a transcription factor which is at least 50% homologous with the transcription factor

- encoded by a DNA sequence comprising the DNA sequence defined in a), or
- iv) encodes a transcription factor which is immunologically reactive with an antibody raised against the purified transcription factor encoded by the DNA sequence defined in a), or
- v) complements the mutation in ToC879, i.e. makes ToC879 able to grow on cyclodextrin and produce lipase when transformed with said DNA sequence.
8. The DNA construct according to any of the claims 5 to 7, in which said DNA sequence is obtainable from a filamentous fungus.
9. The DNA construct according to claim 8, in which said filamentous fungus belongs to any of the groups Phycomycetes, Zygomycetes, Ascomycetes, Basidiomycetes and fungi imperfecti, including Hyphomycetes such as the genera *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium* and *Humicola*, in particular a strain from *Aspergillus* sp., and especially from *A. oryzae*.
10. The DNA construct according to claim 9, in which said DNA sequence is isolated from or produced on the basis of a DNA library of an *Aspergillus oryzae* strain.
11. The DNA construct according to claim 5 to 8, in which said DNA sequence is obtainable from a yeast strain, especially of, *Saccharomyces*.
12. The DNA construct according to claim 7, in which the DNA sequence is isolated from *Escherichia coli* DSM 10666.
13. A recombinant expression vector comprising a DNA construct according to any of claims 5 to 12.
14. A cell comprising a DNA construct according to any of claims 5 to 12, or a recombinant expression vector according to claim 13.

15. The cell according to claim 14, which is a eukaryotic cell, in particular a fungal cell, such as a yeast cell or a filamentous fungal cell.

5

16. The cell according to claim 15, which is a strain of *Aspergillus* sp., in particular a strain of *A. niger* or *A. oryzae*.

10

17. The cell according to claim 15, which is a strain of *Trichoderma* sp., in particular *T. reesei*.

18. The cell according to claim 15, which is a strain of *Saccharomyces*, in particular a strain of *S. cerevisiae*.

15

19. A method of producing a polypeptide of interest comprising growing a cell of any of the claims 14 to 18 under conditions conducive to the production of said factor and said polypeptide of interest, and recovering said polypeptide of interest.

20

20. The method of claim 19, wherein said fungus is a fungus of the genus *Aspergillus*, *Trichoderma*, *Penicillium*, *Fusarium* or *Humicola*.

25

21. The method of claim 20, wherein said cell is of the species *A. oryzae*, *A. niger*, or *A. awamori*.

30

22. The method of claim 19, 20, or 21, wherein said polypeptide of interest is a medicinal polypeptide.

23. The method of claim 22, wherein said medicinal polypeptide is a growth hormone, insulin, or a blood clotting factor.

35

24. The method of claim 19, 20, or 21, wherein said polypeptide is an industrial enzyme.

25. The method of claim 24, wherein said industrial enzyme is a carbonyl hydrolase, carbohydrase, protease, lipase, amylase, cellulase, oxido reductase, glucoamylase, or esterase.
- 5 26. Use of a factor of any of the claims 1 to 4 for enhancing the expression of a polypeptide of interest in a filamentous fungus.
- 10 27. The use of claim 26, wherein said factor is the factor of claim 4.
- 15 28. The use of claim 27, wherein said fungus is a fungus of the genus *Aspergillus*, *Penicillium*, *Trichoderma*, *Fusarium* and *Humicola*, in particular a strain from *Aspergillus* sp., and especially from *A. oryzae* sp.
- 20 29. The use of claim 28, wherein said fungus is of the species *A. oryzae*, *A. niger*, *A. awamori*, *T. reesei*, or *T. harzianum*.
30. The use of any of the claims 26 to 29, wherein said polypeptide of interest is a medicinal polypeptide.
- 25 31. The use of claim 30, wherein said medicinal polypeptide is a growth hormone, insulin, or blood clotting factor.
32. The use of any of the claims 26 to 29, wherein said polypeptide is an industrial enzyme.
- 30 33. The use of claim 32, wherein said industrial enzyme is a carbonyl hydrolase, carbohydrase, protease, lipase, amylase, cellulase, oxido reductase, glucoamylase, or esterase.

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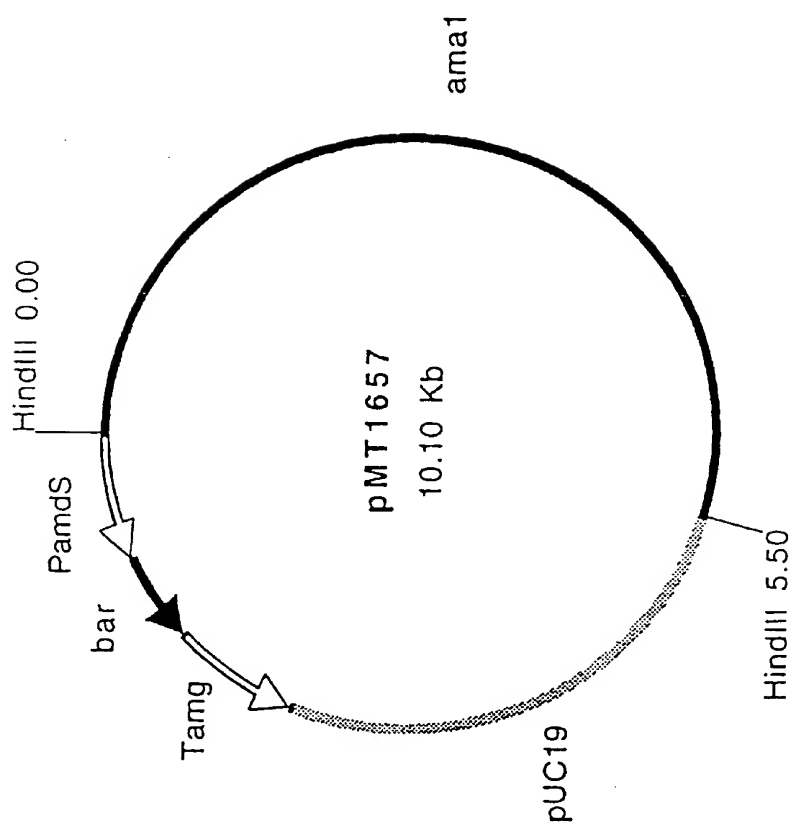


Fig. 1

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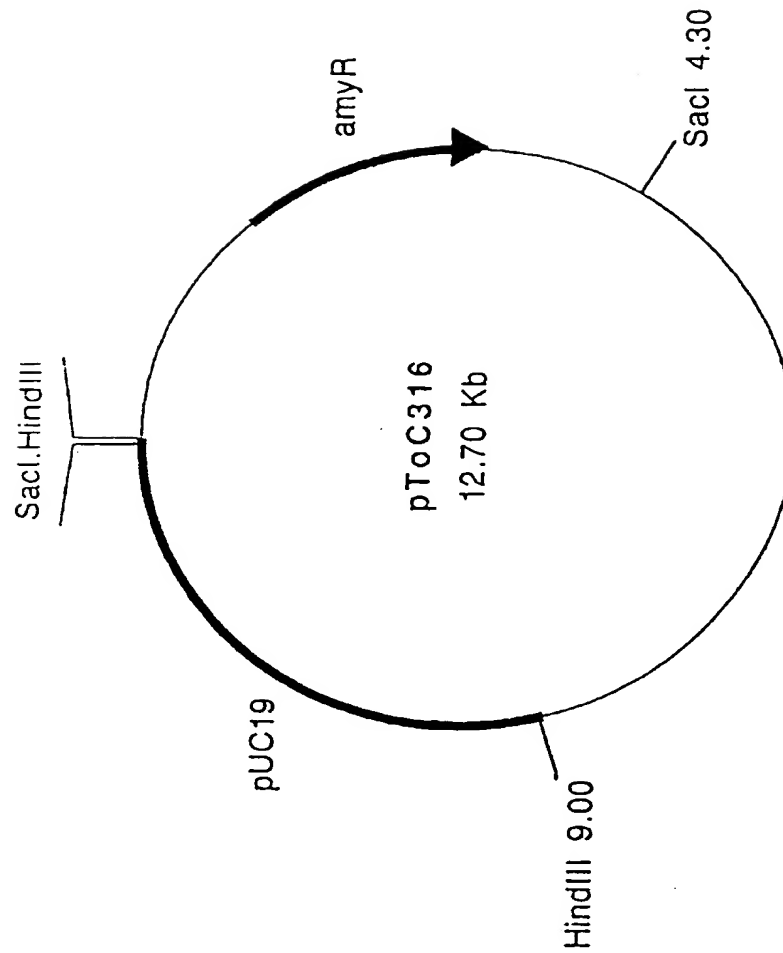


Fig. 2

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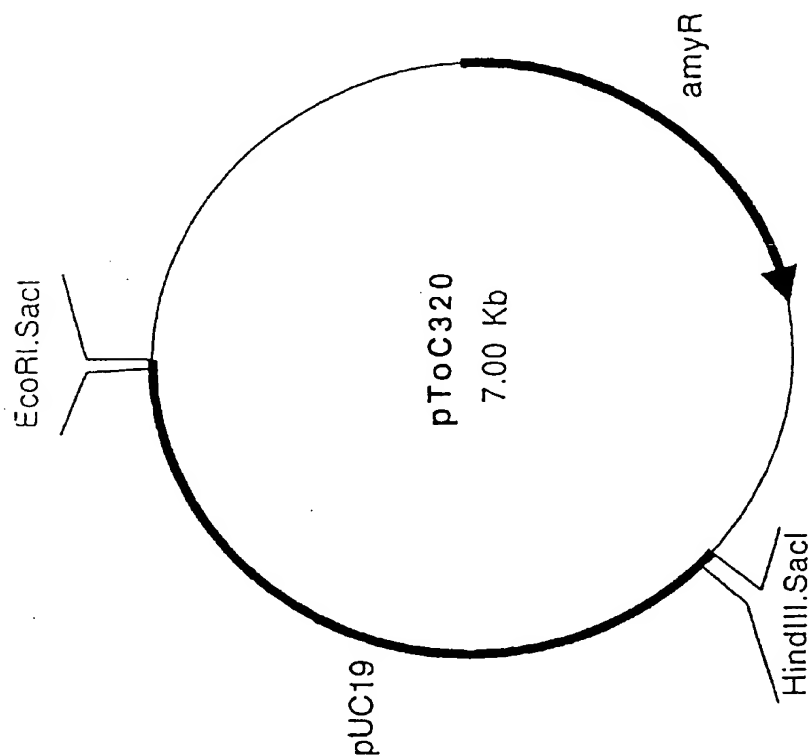


Fig. 3

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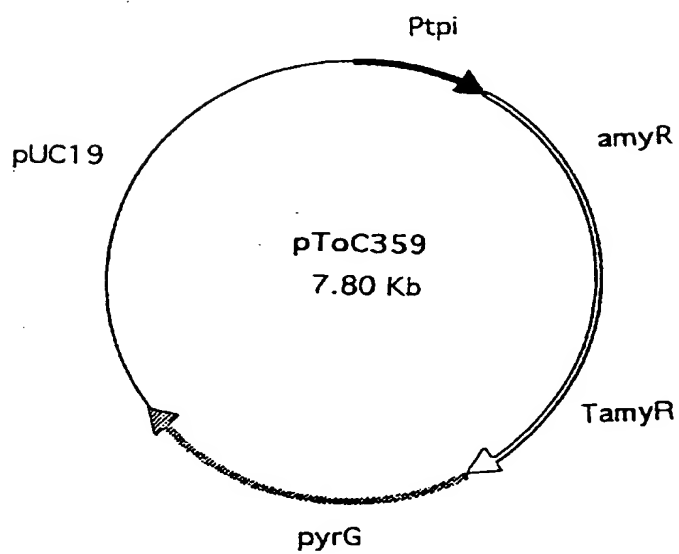
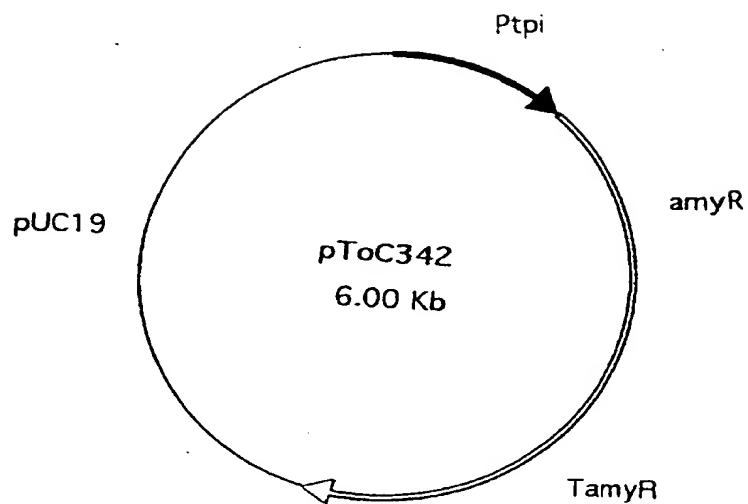


Fig. 4

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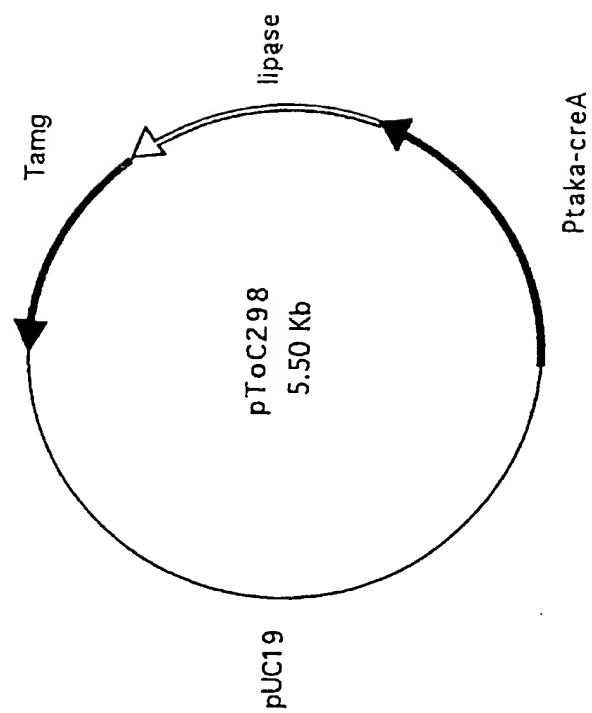


Fig. 5

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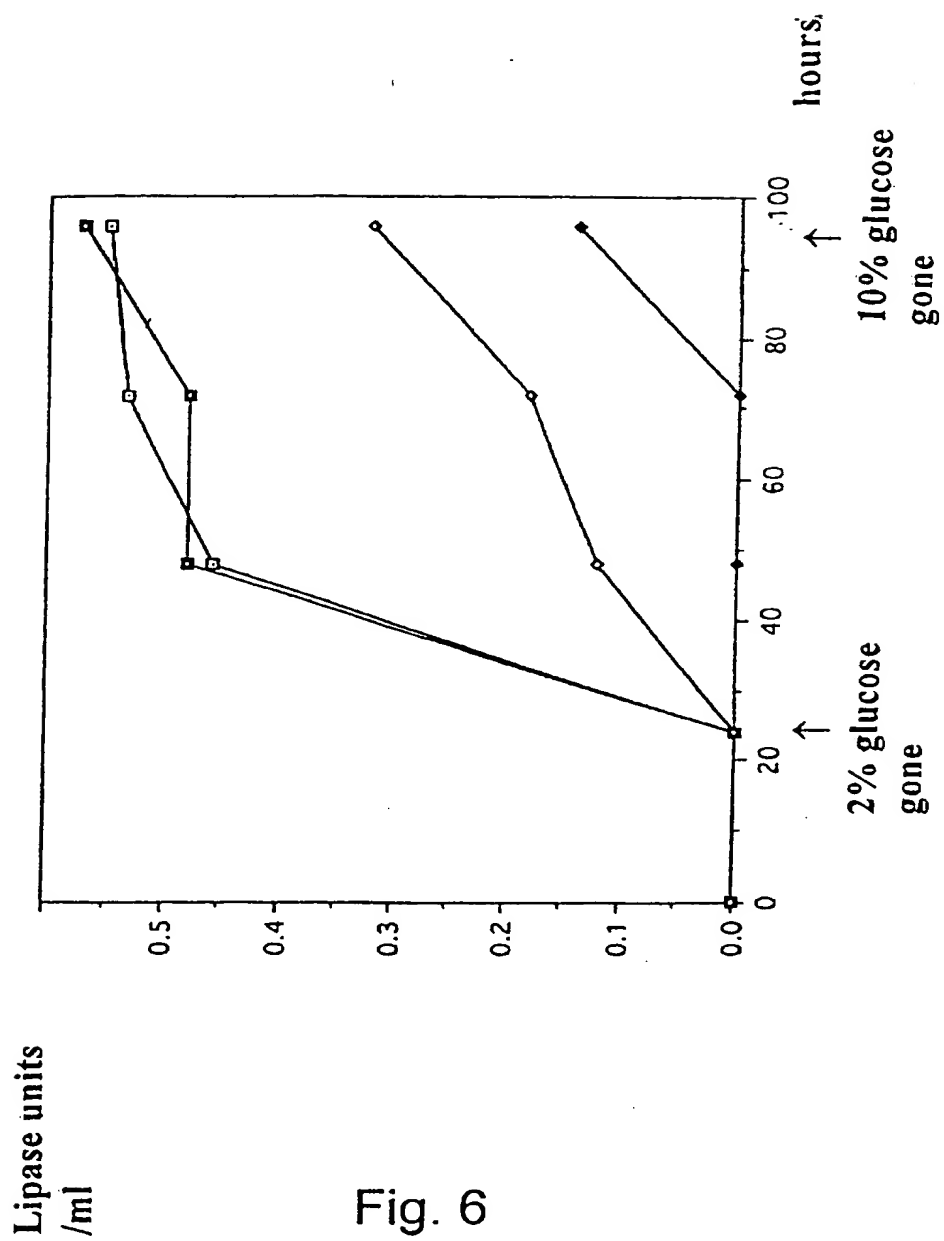
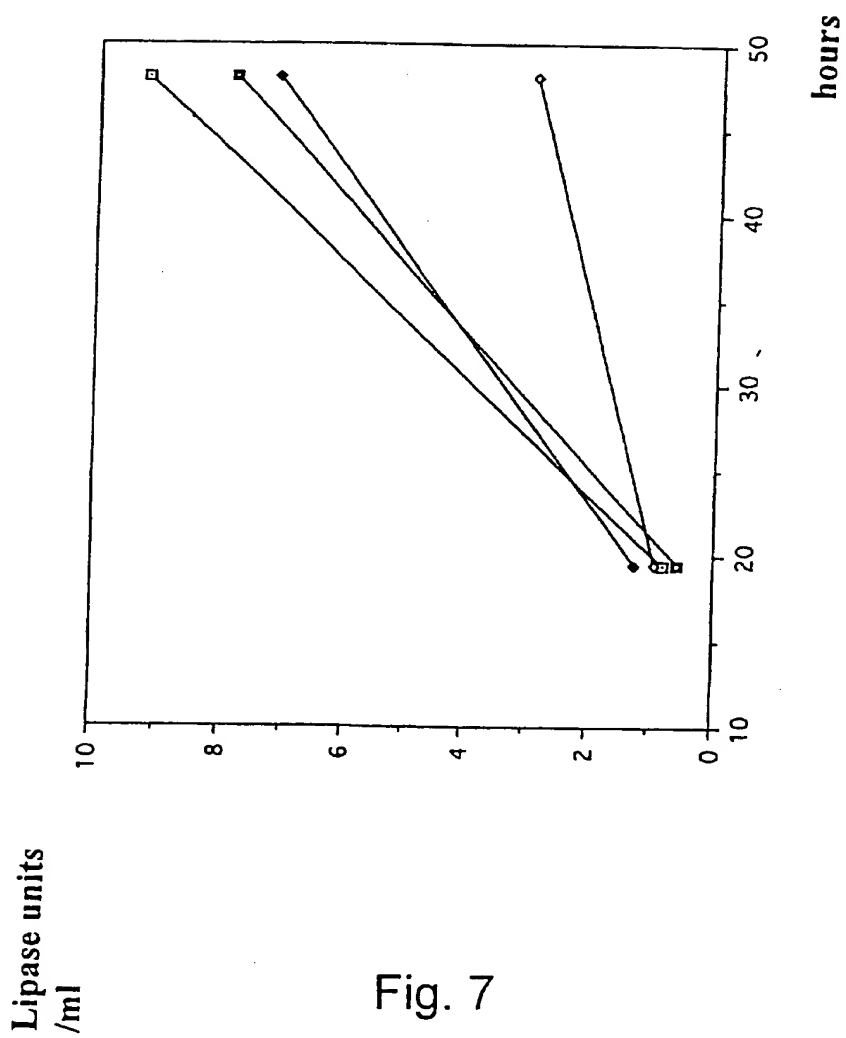


Fig. 6

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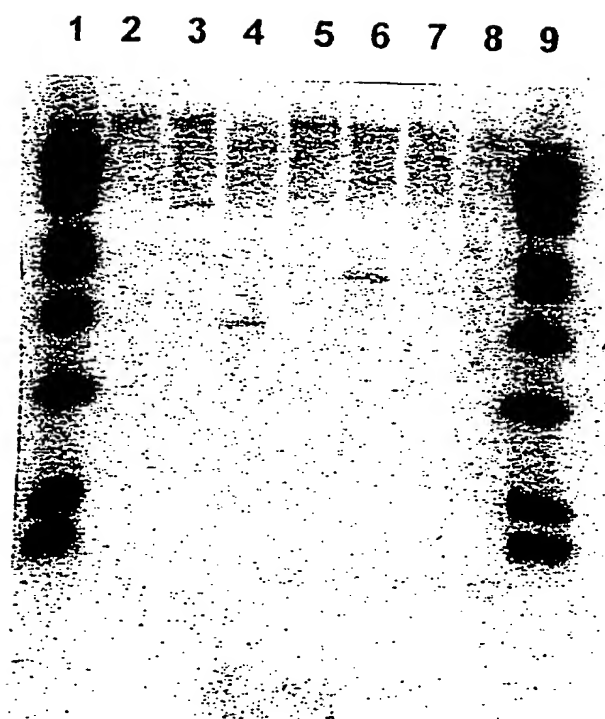


Fig. 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 97/00305

A. CLASSIFICATION OF SUBJECT MATTER		
IPC6: C07K 14/38, C12N 15/80, C12N 1/15 According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC6: C12N, C07K		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
SE,DK,FI,NO classes as above		
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)		
WPI, EDOC, MEDLINE, BIOSIS, DBA, SCISEARCH GENBANK/SWISSPROT/EMBL/DBJ		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Chemical Abstracts, Volume 121, No 12, 19 Sept 1994 (19.09.94), (Columbus, Ohio, USA), Verdoes, Jan C. et al, "The effect of multiple copies of the upstream region on expression of the Aspergillus niger glucoamylase en coding gene", page 272, THE ABSTRACT No 150449j, Gene 1994, 145 (2), 179-187	1-3,5,8-10, 13-18
A		4,6-7,11-12, 19-33
<input checked="" type="checkbox"/> Further documents are listed in the continuation of Box C. <input type="checkbox"/> See patent family annex.		
* Special categories of cited documents: "A" document defining the general state of the art which is not considered to be of particular relevance "B" earlier document but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
29 October 1997		07-11-1997
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86		Authorized officer Patrick Andersson Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00305

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>Dialog Information Services, File 34, SciSearch, Dialog accession no. 13944964, Verdoes JC et al: "Molecular-Genetic Strain Improvement for the Overproduction of Fungal Proteins by Filamentous Fungi", Applied Microbiology and Biotechnology, 1995, V43, N2 (May-Jun), p 195-205</p> <p>---</p>	1-33
A	<p>Dialog Information Service, file 154, Medline, Dialog accession no. 07510263, Medline accession no. 93204901, Nagata O. et al: "Aspergillus nidulans nuclear proteins bind to a CCAAT element and the adjacent upstream sequence in the promoter region of the starch-inducible Taka-amylase A gene", Mol Gen Genet (GERMANY) Feb 1993, 237 (1-2) p251-60</p> <p>---</p> <p>-----</p>	1-33

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00305

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
2. ☒ Claims Nos.: 1, 7 and related claims
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

see next page
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
☐ No protest accompanied the payment of additional search fees. - - - - -

Form PCT/ISA/210 (continuation of first sheet (1)) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.

PCT/DK 97/00305

The wording of claim 1 "A transcription factor regulating the expression of an alpha amylase promoter in filamentous fungus" is not clear as promoters are not expressed i.e. the claim does not fulfill the prescribed requirements of a claim see Art 6 and Art 17(2)(a)(ii). The claim has been interpreted as " A transcription factor regulating alpha amylase promoter initiated expression in filamentous fungus"

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